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ELIMINATION OF C^{14}-LABELED THYROXINE IN THE BILE, URINE AND EXPIRED AIR OF RATS IN ALTERED THYROID STATES[1]

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ABSTRACT

Adult male Sprague-Dawley rats were treated with appropriate measures to attain the desired metabolic states. DL-thyroxine-1-C^{14} was administered subcutaneously and the elimination of radioactivity determined in bile, urine, and expired air of hypo-, hyper- and euthyroid animals.

Biliary elimination of C^{14} activity in the control rats amounted to 11.8% of the injected dose within a period of six hours. Desiccated thyroid treated animals showed an increased excretion of radioactivity accompanied with an increased volume of bile produced. The thyroidectomized and thiouracil-treated rats showed no change from normal in the amount of radioactivity eliminated but both groups had a reduction in the volume of bile removed.

Urinary excretion of thyroxine activity during the twelve hour period never exceeded 2% of the injected amount regardless of the group studied. Hyperthyroid animals showed increased urinary excretion of radioactivity with increased volume of urine eliminated. The C^{14} activity in the urine of the thyroidectomized and thiouracil-treated groups was lower than the control animals but only significantly so in the latter case. This reduction in the amount of activity was found to be independent of the urine volume excreted.

The recovery of C^{14} in the expired air was taken to indicate that decarboxylation of the administered thyroxine had occurred. Within twelve hours about 12% of the injected activity appeared as $C^{14}O_2$ in the control group. No alteration in this amount was observed in the hyperthyroid animals. Both hypometabolic groups showed decreased $C^{14}O_2$ production, however, this was only significant in the thiouracil-treated animals.

WITHIN the last two decades I^{131}-labeled thyroxine has been used extensively in studying the degradation and elimination of the thyroxine molecule in both animals and humans. Because the detection of I^{131} activity does not always indicate the presence of the intact thyroxine

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molecule without further chemical analysis, abnormally high levels of excretion for this compound have appeared in the literature.

With the synthesis of C^{14}-carboxyl labeled thyroxine (1), it is now possible to make use of another radioactive element in following the metabolism of the thyroxine molecule; the use of this element furthermore affords an opportunity to study an additional mechanism in the degradation of this compound, namely that of decarboxylation.

The present study was carried out primarily to compare our results with those previously reported using I^{131}-labeled thyroxine, and secondly, to investigate the effect of altered metabolic states on the metabolism of DL-thyroxine-1-C^{14}.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain were used throughout the investigation. Animals were divided into four groups comprising a normal, a hyperthyroid and two hypothyroid groups and were treated accordingly to attain the desired metabolic status.

Normal animals were maintained on a Rockland Complete Rat Diet. A hypermetabolic rate was achieved from a diet consisting of 3 gm. desiccated thyroid/Kg. of finely ground stock food. *Ad libitum* feeding on this regime for 3 weeks resulted in the achievement of the desired hypermetabolic level.

For thiouracial feeding, the animals were fed a diet containing the drug concentration of 0.2 gm.%. Animals were maintained on this regime until the metabolic rate decreased about 25%.

Thyroidectomies were performed under ether anesthesia and the animals were maintained on stock diet until ready for use.

A Benedict-Roth closed circuit apparatus was employed for the measurement of oxygen consumption. Basal metabolic rates were obtained by measuring oxygen uptake over three consecutive thirty-minute intervals. Resting metabolic rates of the control group ranged from 90 to 110 cc. 0_2/100 gm./hr. An increase or decrease of 25% in oxygen consumption above or below the control levels was used as the criterion for designating animals hyper or hypometabolic.

DL-thyroxine-1-C^{14}, which was used throughout this investigation,[2] had a specific activity of 5.3 μg./mg. All radioactivity measurements were corrected for self-absorption and background and converted into μgm. of thyroxine, by reference to the known specific activity of the administered C^{14} thyroxine. Samples were counted to within a probable error of 3 to 4% in the bile study and to within 6 to 7% in the $C^{14}O_2$ and urine studies. By way of illustration a 200 gram rat received about 840,000 cts./min. and a 0.1 ml. bile sample would have about 1000 cts./min. or 50 times background. All injections were made subcutaneously at a dose level of 780 μg./Kg. body weight. Because of the problem of counting the weak beta radiation from carbon 14, a pharmacological rather than physiological dose was employed in this study. At this dose level certain alterations in the metabolism of the excretory tissues may have occurred if the study had been extended for a longer period of time. However, because this study was limited to 6 hrs. for the collection of bile and 12 hrs. for the urine and $C^{14}O_2$ collections any changes in the metabolism of the excretory tissues was probably not a result of the administered dose of labeled material but by previous treatment.

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[2] The C^{14} for the synthesis of the thyroxine was obtained on allocation from the U.S. Atomic Energy Commission.

Bile samples were obtained by cannulating the common bile duct with polyethylene tubing (inside diameter 0.028 cm.). When a good rate of flow was obtained, the animal was placed in a restraining cage and injected with labeled thyroxine. Aliquots of 0.1 ml. of hourly bile samples were plated in duplicate on copper planchets and diluted with distilled water and 95% alcohol to obtain even distribution of the material and were then allowed to air dry.

For 12-hour urine collections, animals were placed in metabolic cages. The concentration of C^{14} activity in the urine was determined on 0.1 ml. aliquots of samples obtained at 3 hour intervals. The techniques involved in plating, drying, and counting were as described for bile.

Carbon dioxide collections were made using the modified Benedict-Roth closed circuit apparatus (2). The collections were made at 2 to 3 hour intervals through a period of 12 hours. The quantity of radioactivity in each aliquot was then determined using a windowless flow counter.

RESULTS

Table 1 shows the percentage of C^{14}-activity excreted and volume of bile produced. At the end of 6 hours normal animals excreted 11.80% of the injected dose of radioactivity via the biliary route. The hypothyroid groups did not differ significantly from the normal in this respect. Mean excretion values of C^{14}-activity in the thyroidectomized and thiouracil treated animals were 12.04% ($P=0.953$) and 11.90% ($P=0.940$) respectively. A decrease in the volume of bile produced was shown by the thyroidectomized group while no significant alteration in volume was seen in the thiouracil-treated animals.

The amount of radioactivity excreted in the bile of animals treated with desiccated thyroid was significantly elevated ($P=0.001$) above normal, reaching a level of 21.66%, 6 hours after injection. Associated with this increased elimination of radioactivity was a rise in bile production to a level $1\frac{1}{2}$ times greater than normal.

In Table 2 are presented the percentages of injected radioactivity excreted and volume of urine produced for the various groups. In neither group was the level of urine radioactivity above 2% at the end of a 12-hour interval. Normal animals excreted 1.15% of the injected C^{14} activity in 6.22 ml. of urine during the 12-hour collection period. The thiouracil

TABLE 1. CUMULATIVE 6-HOUR BILIARY EXCRETION FOLLOWING SUBCUTANEOUS INJECTION OF C^{14}-LABELED THYROXINE

Condition	No. of animals	% activity excreted[a]	P[b]	ml. bile excreted	Mean BMR (cc. O_2/100 gm./hr.)
Normal	7	11.80 ± 3.42	—	4.31	90–110[c]
Desiccated Thyroid-Treated	6	21.66 ± 3.52	0.001	7.10	169.9
Thyroidectomized	6	12.04 ± 9.07	0.953	2.73	57.9
Thiouracil-Treated	9	11.90 ± 2.09	0.945	3.96	68.6

[a] Mean ± standard deviation.
[b] Probability of no difference from normal.
[c] Range of values for normal animals.

TABLE 2. CUMULATIVE TWELVE-HOUR URINARY EXCRETION FOLLOWING SUBCUTANEOUS INJECTION OF C^{14}-LABELED THYROXINE

Condition	No. of animals	% activity excreted[a]	P[b]	ml. urine excreted	Mean BMR (cc. O_2/100 gm./hr.)
Normal	10	1.15 ± 0.25	—	6.22	90–110[c]
Desiccated Thyroid-Treated	8	1.89 ± 0.40	0.001	11.95	154.8
Thyroidectomized	6	1.07 ± 0.57	0.688	6.4	68.1
Thiouracil-Treated	9	0.79 ± 0.31	0.008	6.02	64.4

[a] Mean ± standard deviation.
[b] Probability of no difference from normal.
[c] Range of values for normal animals.

treated group showed a significant decrease in the amount of radioactivity eliminated (P = 0.008) while the thyroidectomized group did not (P = 0.688.) The quantity of radioactivity appearing in the urine of hyperthyroid animals was significantly increased to 1.89% (P = 0.001). The volume of urine produced by the hypermetabolic group was almost double (11.9 ml.) that of the normal (6.2 ml.) while neither of the hypothyroid groups showed a noticeable change in urine volume when compared with the control group.

In Table 3 are presented the mean cumulative percentages of $C^{14}O_2$ in the expired air by the various groups. This table shows that both hypometabolic groups excreted less $C^{14}O_2$ in 12 hours than the euthyroid animals. Thyroidectomized and thiouracil treated animals show a mean excretion of 4.04 and 2.67%, respectively, with a significant decrease (P = 0.006) present in the latter group only. During the 12 hour collection period hypermetabolic animals exhibited no alteration (P = 0.992) in eliminating $C^{14}O_2$ when compared with the euthyroid group.

DISCUSSION

The fact that the liver plays an important role in the metabolism of thyroxine is born out by the predominance of radioactivity appearing in the bile. The mean excretion of 11.80% of the C^{14}-activity in the bile of normal animals agrees closely with the results reported earlier when using I^{131} labeled L-thyroxine (3) and C^{14}-labeled DL-thyroxine (2). Other in-

TABLE 3. CUMULATIVE TWELVE-HOUR $C^{14}O_2$ EXCRETION FOLLOWING SUBCUTANEOUS INJECTION OF C^{14}-LABELED THYROXINE

Condition	No. of animals	% activity excreted[a]	P[b]	Mean BMR (cc. O_2/100 gm./hr.)
Normal	6	6.18 ± 2.39	—	90–110[c]
Desiccated Thyroid-Treated	8	6.20 ± 2.47	0.992	186.6
Thyroidectomized	5	4.04 ± 0.78	0.288	71.8
Thiouracil-Treated	7	2.67 ± 1.26	0.006	68.6

[a] Mean ± standard deviation.
[b] Probability of no difference from normal.
[c] Range of values for normal animals.

vestigators (4) gave a comparable value of about 13% excretion via this route in 6 hours using tracer amounts of L-thyroxine I^{131}. It was reported (5), that much of the I^{131} which appeared in the bile was in the form of a glucuronide of thyroxine.

The marked increase in the biliary radioactivity of hypermetabolic animals may be due to increased liver metabolism. A previous report (3) indicated that there was no significant increase in I^{131} activity in the bile of hyperthyroid animals having been injected with L-thyroxine. One possible explanation for this discrepancy may be due to the difference in turnover rates of the L and D isomers of thyroxine. It has been reported (6) that after a physiological or pharmacological dose of L-thyroxine the resulting iodinemia is reduced to 1 to 2% of the initial level within 2 hours. Also the disappearance from the blood of both of the thyroxine isomers have been reported (7) to be composed of five exponential decay rates which can be reduced to four by drainage of the bile to the outside or removal of the thyroid gland. The first three rates are the same for both isomers, however, the half life of the final rate for the L-isomer is between 2 to 3 times that for the D form indicating a more rapid turnover of the D isomer. Other investigations have shown that the metabolism of a thyroidectomized rat can be maintained at its normal level by the administration of 12 μg. DL-thyroxine per Kg. of body weight/day (8), while only 6 μg. of L-thyroxine is required per Kg. per day (9).

Although there is a diminution of the bile production by hypothyroid animals, this appears to be only a characteristic of the hypometabolic state and is not reflected in the amount of radioactivity excreted.

Apparently thyroidectomized and thiouracil-treated animals suffer no loss in the ability of the liver to metabolize exogenous thyroxine at this dose level although bile production is reduced. It has been observed (10) that hyperthyroid animals produce approximately twice the volume of bile per day when compared with hypothyroid animals. Such a relationship was also found in the present study.

The urinary pathway appears to play a minor role as a route of excretion for the C^{14}-labeled thyroxine molecule per se. Although the percentage of radioactivity excreted in the urine was low in all groups, there was a statistically significant increase above the normal level of 1.15% found in the hyperthyroid animals after 12 hours. C^{14}-activity in the urine of both thyroidectomized and thiouracil-treated animals was lower than the control group but only significantly so in the latter.

Earlier reports (6, 11) indicated that in a period of 24 hours normal rats excreted between 11.0% and 18.2% of the injected I^{131}-labeled thyroxine in the urine. Analysis of the urine samples showed the majority of the I^{131} to be in the form of inorganic iodide. Intact, radioactive thyroxine usually appeared in amounts of less than 5% of the total I^{131} excreted in the urine.

The apparent discrepancy that exists with regard to the quantity of

radioactivity appearing in the urine following administration of C^{14}-labeled and I^{131}-labeled thyroxine is probably the result of large quantities of I^{131} being excreted as inorganic iodide resulting from metabolized I^{131}-labeled thyroxine and not representative of the intact thyroxine molecule.

Although the differences in the amount of radioactivity recovered in the urine of the hyperthyroid and thiouracil treated animals were small when compared to the control group, the values obtained were highly significant ($P = 0.001$ and $P = 0.008$, respectively). The observed differences are likely due to changes in metabolism, which allows increasing or decreasing amounts of available metabolites to be excreted. Thyroidectomy appears to have no effect on the excretion of thyroxine or its metabolites in the urine. Urine production itself does not seem to affect the amount of radioactivity that will be excreted by this route.

The recovery of C^{14} in the expired air indicates that catabolism of the thyroxine molecule has taken place in the body, and that this pathway may be an important route for the elimination of thyroxine metabolites.

Both hypometabolic groups show a decrease in elimination of $C^{14}O_2$ as shown in Table 3. There is, however, a significant alteration only among the thiouracil-treated animals. It would seem safe to conclude that the rate of decarboxylation of the thyroxine molecule is decreased with lowering of the metabolic state of the animal below a critical level of about 70cc. O_2/100gm./hr.

The hypermetabolic animals and the control group eliminated nearly identical amounts of $C^{14}O_2$ after 12 hours. A much higher rate of $C^{14}O_2$ excretion was previously reported in normal animals (2). After a similar time interval they found 10% of the injected C^{14} activity to be eliminated by this route as compared to 6.2% in this report.

The fact that hyperthyroid animals should show no increase in $C^{14}O_2$ may be due to one of two factors. The high biliary elimination of C^{14} activity may have lowered the amount of available thyroxine or its metabolites to be eliminated by other routes or with an increased body metabolism there may have been little or no increase in the amount of the decarboxylating enzymes.

In summarizing the differences found in various metabolic states the hyperthyroid animals showed an increased biliary excretion, of what may be conjugated thyroxine with an increased volume of bile produced. There was a small but significant increase in urinary excretion of thyroxine, but no significant alteration in the rate of decarboxylation of thyroxine as measured by the appearance of $C^{14}O_2$ in the expired air.

Neither of the hypometabolic groups showed an alteration in the amount of thyroxine eliminated in the bile although both groups had a reduction in the volume of bile produced. In both groups the urinary excretion was reduced, but only significantly in the thiouracil-treated animals, with no significant change in the volume of urine eliminated. Re-

duced decarboxylation of thyroxine was found in both hypometabolic groups although this was only highly significant in those animals receiving thiouracil.

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EFFECT OF THYROID IMBALANCE ON THE DETOXICATION OF BENZOIC ACID IN THE RAT

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ABSTRACT

Thyroid hormone imbalance has been shown to profoundly influence the detoxication of benzoic acid in the rat. The excretion of hippuric acid and benzoylglucuronide following the administration of sodium benzoate is low in hyperthyroid rats when compared with normal. *In vitro* studies with tissue slices have confirmed the results obtained with intact rats. The thyroid status of the animal has also been found to influence the utilization of administered glucuronolactone. The β-glucuronidase activity of hypothyroid rat livers is depressed below normal and the extent of glucosiduronic acid synthesis in these livers is much higher than normal. The various implications of these results are discussed.

THE study of the detoxication as well as the metabolism of foreign organic substances in the animal body has, in recent years, developed into an elegant and recognised method of investigating the mechanism of several synthetic processes in the body. Of the diverse metabolic reactions that are altered under thyroid stress, the influence of thyroid hormone on oxidative phosphorylation has received critical attention in recent years. Though Tapley, Cooper and Lehninger (1) have recently observed that *in vitro*, thyroxine has no direct influence on oxidative phosphorylation processes, the same authors and several others (2, 3, 4) have shown that thyroid hormone *in vivo* depresses oxidative phosphorylation. Ball and Cooper (5) have recently outlined the probable mechanism whereby, in hyperthyroidism, phosphorylation would be depressed and oxygen consumption enhanced.

Benzoic acid is normally metabolized in the body to form hippuric acid, a glycine conjugate, and also as benzoylglucuronide. Recent investigations have shown that hippuric acid synthesis (6) and glucuronide conjugation (7) are both dependent on high energy phosphates. Burlington (8) studied the influence of endocrines on p-amino hippuric acid synthesis by rat liver homogenates and showed that the ability of the homogenates to synthesize p-amino hippuric acid is altered by adrenalectomy, a condition of alloxan diabetes (representing the influence of insulin lack) and

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ovariectomy. Fatterpaker, Marfatia and Sreenivasan (9) have observed that feeding iodinated casein to rats reduces the hippuric acid synthesis by liver homogenates.

A detailed investigation into the influence of thyroid stress on benzoic acid detoxication has revealed the two endergonic processes—hippuric acid and benzoylglucuronide synthesis—to be profoundly influenced by the thyroid status of the animal. The present study also provides indirect evidence for the participation of high energy phosphate (uridine diphosphate glucuronic acid) in the synthesis of glucosiduronic acids.

EXPERIMENTAL

Animals:

Albino rats weighing between 150 and 160 gm. were used in the present series of experiments. Each series consisted of rats divided into 3 groups: the control group fed the laboratory stock diet, the hypothyroidal group fed the stock diet plus a supplement of 50 mg. of methylthiouracil per rat per day, and the hyperthyroid group fed the stock diet plus 16 mg. of desiccated thyroid power (B.D.H.) per rat per day. In each set of experiments, a total of not less than fifteen rats was used. The diets were fed *ad libitum* and sufficient distilled water was given. Vitamin A and D were given as a commercial preparation. Each rat received per week 7200 I.U. of vitamin A, 1200 I.U. of vitamin D, 2 mg. of α-tocopherol and 0.2 mg. of vitamin K. From preliminary experiments (10) it was observed that for hypo- and hyperthyroidal conditions to set in completely, a six-week period of feeding on the above regimen was necessary.

Benzoic acid detoxication:

In preliminary experiments reported earlier (10), benzoic acid was fed orally to rats. In the present study, however, it was found convenient to administer benzoic acid by intraperitoneal injection as sodium benzoate. Each rat received a single injection of 50 mg. of benzoic acid. The rats were transferred to metabolism cages and the urine of the subsequent 48 hours was collected in 1 ml. of 1N H_2SO_4 to which a few drops of toluene were added as a preservative. The urine sample in each case was made to 100 ml., filtered and preserved under toluene in an ice-chest.

Hippuric acid in the samples was estimated by the paper chromatographic procedure of Gaffney, Schreier, Di Ferrante and Altman (11). 100 μl. of the urine samples and known amounts of pure hippuric acid were chromatographed on Whatman No. 1 paper in the solvent system, n-butanol:glacial acetic acid:water (4:1:1 by volume). After drying, the chromatogram was sprayed with a 4% solution of p-dimethylaminobenzaldehyde in acetic anhydride containing a few crystals of sodium acetate, and again dried at 130° C. The orange-colored hippuric acid-azlactone spots (R_f 0.80) were then cut out and eluted thrice with 2 ml. lots of methanol. The intensity of the color was measured at 460 mμ in a Beckman model DU spectrophotometer.

Benzoylglucuronide in the urine samples was estimated by the method of Bray, Humphris, Thorpe, White and Wood (12). Suitable aliquots were transferred to boiling tubes, the volume in each tube was made to 2 ml. and to each tube were added 4 ml. of conc.HCl and 2 ml. of 0.375% naphthoresorcinol solution. The tubes were heated in a boiling water bath for 2 hours. After cooling, 5 ml. of n-butanol was added to each tube and the contents mixed well. After about 2–3 minutes the color was read in a Lumetron Photoelectric Colorimeter (Photovolt Corporation) employing yellow-green (530 mμ) filter.

Metabolism of glucuronolactone:

This experiment was conducted with a fresh set of rats rendered hypo- and hyperthyroidal as above. Each rat after an intraperitoneal injection of 100 mg. of glucuronolactone was transferred to a metabolism cage and the urine collected during the next 24 hours in 2 ml. of 1N H_2SO_4 to which a few drops of toluene were added. The total glucuronic acid was estimated according to the method of Bray and coworkers (vide supra).

In vitro synthesis of ortho-aminophenol glucuronide (OAP-glucuronide) by liver slices:

The same set of rats used for the above experiment was used in the present investigation. After the injection of glucuronolactone, a lapse of 10–15 days was allowed for the rats to reach their respective normal status, after which they were killed. The method followed for glucuronide synthesis was essentially that of Levvy and Storey (13). The reaction was carried out in 25 ml. conical flasks in a Dubnoff metabolic shaking incubator for a period of 90 minutes at a bath temperature of 37° C. The composition of the reaction medium is given in detail in Table 4. After removing the slices for dry weight and nitrogen determination, 2 ml. of a fresh mixture of equal volumes molar trichloroacetic acid and molar phosphate was added to the flasks. After centrifuging, suitable aliquots of the supernatant were removed for color development by diazotisation and coupling with naphthethylene diamine dihydrochloride. The color complex was measured in a Beckman spectrophotometer (model DU) at 550 mμ. Each liver sample was tested in triplicate to determine its ability to synthesize OAP-glucuronide.

Liver β-glucuronidase activity:

The enzyme extract from the individual liver samples was prepared according to the method of Kerr, Graham and Levvy (14). To a cell-free liver homogenate (1%) was added 0.3 *M* citrate buffer pH 5.2 to remove the inactive proteins and the supernatant after centrifugation was treated with an equal volume of saturated ammonium sulfate solution, centrifuged again and the residue dissolved in water. This solution was treated as the enzyme source.

The incubation mixture for β-glucuronidase assay contained 4 ml. of 0.1 *M* acetate buffer pH 4.5, 0.5 ml. of 0.01 *M* sodium phenolphthalein glucuronide (in the control the substrate was added at the termination of the incubation period) and 0.5 ml. of the appropriate enzyme solution. The incubation was carried out at 37° C for a period of 60 minutes. The reaction was stopped by the addition of 5 ml. of 0.4 *M* glycine buffer pH 10.45. The intensity of the color formed due to the liberation of free phenolphthalein by the action of the enzyme was measured in a Lumetron colorimeter using filter 530 mμ. The substrate consisting of 0.01 *M* sodium phenolphthalein glucuronide was prepared from the pure cinchonidine salt of phenolphthalein monoglucuronide according to the method of Talalay *et al.* (15).

RESULTS

In Table 1 are presented data regarding the excretion of hippuric acid and benzoyl glucuronide, by rats under thyroid stress, following an intraperitoneal injection of 50 mg. of benzoic acid. It can be seen that the excretion of both hippuric acid and benzoyl glucuronide is depressed to a marked extent in hyperthyroidal rats, whereas it is enhanced in hypothyroidal animals. The resuls presented in Table 2 give a comprehensive picture of the extent of detoxication of benzoic acid in various states of

TABLE 1. INFLUENCE OF THYROID HORMONE ON HIPPURIC ACID AND BENZOYLGLUCURONIDE EXCRETION IN THE RAT

Condition	No. of rats	Hippuric acid excretion* (mg.)	Percent deviation from normal	Benzoyl glucuronide excretion* (mg.)	Percent deviation from normal
Normal	5	27.7± 1.4† (26.6–30.6)	—	16.6± 1.9† (13.5–20.0)	—
Hypothyroid	5	40.8± 8.0 (28.0–63.9)	+47	22.4± 4.5 (18.0–36.5)	+35
Hyperthyroid	5	13.1± 1.8 (11.0–16.7)	−53	10.2± 2.0 (7.3–12.3)	−39

* The values in parenthesis indicate range.
† Standard deviation.

TABLE 2. INFLUENCE OF THYROID HORMONE ON DETOXICATION OF BENZOIC ACID

Condition	Hippuric acid expressed as benzoic acid (mg.)	Benzoyl glucuronide expressed as benzoic acid (mg.)	Total amount of benzoic acid derivatives excreted (mg.)	Percent of injected benzoic acid excreted as conjugates
Normal	18.86	6.80	25.66	51
Hypothyroid	27.90	9.17	37.07	74
Hyperthyroid	8.93	4.17	13.10	26

TABLE 3. METABOLISM OF GLUCURONOLACTONE IN NORMAL, HYPO- AND HYPERTHYROIDAL RATS
(100 mg. of glucuronolactone injected intraperitoneally)

Condition	Number of rats	Glucuronic acid excreted in 24 hours (mg.)		Per cent deviation from normal
		Range	Mean	
Normal	5	28–56	35±7.2*	—
Hypothyroid	5	18–38	26±7.4	−26
Hyperthyroid	5	45–64	53±5.7	+51

* Standard deviation.

thyroid stress, the amounts of benzoic acid conjugates excreted being expressed in terms of benzoic acid.

The influence of thyroid hormone on glucuronide conjugation was considered worth studying in detail, since it is known that both thyroxine and triiodothyronine are excreted from the liver as glucuronide conjugates via the bile into the intestinal tract (16). In Table 3 is shown the excretory pattern of total glucuronic acid following an intraperitoneal injection of 100 mg. of glucuronolactone to the three groups of rats. The results show that the excretion is depressed considerably in hypothyroidism, whereas, it is enhanced by an appreciable extent in hyperthyroidism.

TABLE 4. EFFECT OF THYROID HORMONE IMBALANCE ON 'IN VITRO' SYNTHESIS OF O-AMINOPHENOL GLUCURONIDE IN RAT LIVER SLICES

The reaction medium was of the following composition: 2 ml of bicarbonate-Ringer medium containing 0.02 *M* lactate, 0.001 *M* ascorbic acid and 0.0025% o-aminophenol. The bicarbonate-Ringer medium did not contain any sulphate as suggested by Levvy and Storey (14). Liver slices of 20 mg. wet weight (approximately) per flask. Incubation for a period of 90 minutes at 37° C. Gas phase 95% oxygen and 5% carbon dioxide.

Condition	Number of rats	O-aminophenol glucuronide synthesized per mg. nitrogen of liver tissue in 90 minutes (μg.)		Per cent deviation from normal
		Range	Mean	
Normal	5	20.87–29.66	24.64 ± 3.0*	—
Hypothyroid	5	30.50–37.20	33.48 ± 2.4	+36
Hyperthyroid	5	18.90–20.30	19.80 ± 0.5	−20

* Standard deviation.

The results presented in Table 4 show that the *in vitro* synthesis of OAP-glucuronide by liver slices of rats under thyroid stress, follows the same pattern as the *in vivo* synthesis of benzoyl glucuronide. In Table 5 are summarized the results of assay for β-glucuronidase activity in liver samples of eu-, hypo- and hyperthyroid rats. The enzyme activity is considerably enhanced in hyperthyroidism, whereas, it is decreased in hypothyroidism.

DISCUSSION

The work of Chantrenne (17) and more recently that of Schachter and Taggart (18) have demonstrated the essential nature of adenosinetriphosphate (ATP) in the formation of benzoyl coenzyme A, the active coupling agent in the hippuric acid conjugating system. Dutton and Storey (19) and Dutton (20) have similarly shown uridinediphosphate glucuronic acid (UDPGA), a high energy phosphate compound to be an active participant in the synthesis of ether- and ester-linked glucuronides. Thus, a decreased synthesis of benzoylglucuronide and benzoylglycine in hyperthyroidism is to be expected, if, as suggested by recent workers (2–5), the hyperthyroidal state is associated with decreased phosphorylation and a consequent poor availability of high energy phosphate compounds.

TABLE 5. β-GLUCURONIDASE ACTIVITY OF LIVERS OF NORMAL, HYPO- AND HYPERTHYROIDAL RATS

Condition	Number of rats	β-glucuronidase activity per gm. of liver*		Per cent deviation from normal
		Range	Mean	
Normal	4	3.94–5.40	4.71 ± 0.47†	—
Hypothyroid	5	2.51–3.34	3.07 ± 0.21	−35
Hyperthyroid	5	6.31–8.57	7.65 ± 0.59	+62

* Arbitrary unit of β-glucuronidase is defined as mg. of phenolphthalein liberated from phenolphthalein glucuronide in an hour at 37° C by 1 gm. of fresh liver tissue.
† Standard deviation.

Hyperthyroidism is associated with an increased catabolism of carbohydrate and in this condition tissue vitamin C is decreased (21, 22) and the requirement for vitamin C is greater (23). The opposite has been shown to hold for hypothyroidism (21). Glucuronic acid is known to be a common precursor for both ascorbic acid and glucosiduronic acid. Since carbohydrate metabolism proceeds at different rates in the various states of thyroid stress, the excretory pattern of glucuronolactone indicated in Table 3 may well reflect the extent of utilization of the same for various metabolic needs of the body. The results presented in Table 3 are in keeping with the enhanced synthesis of benzoylglucuronide observed in hypothyroidal rats in a separate experiment (Table 1) and would seem to suggest that in hypothyroidal condition, the utilization of glucuronolactone is better than in normal, the retained amount being utilized at least in part for the formation of increased amounts of tissue vitamin C and the glucuronide conjugates. On the contrary, in hyperthyroidism the utilization seems to be poor, even the glucuronic acid retained in the system probably being mainly catabolized. Thus, the present results suggest that the lowered excretion of glucuronide observed in hyperthyroidism may be the result of *the combined effect of a reduced availability and lowered mobilization of glucuronic acid for conjugation and a decreased rate of conjugation due to the lack of high energy phosphate compounds.* The pattern of results obtained in the course of the study of the *in vitro* synthesis of OAP-glucuronide by liver slices of rats under thyroid stress (Table 4) confirms the *in vivo* observation.

Dutton and Storey (19) and Isselbacher and Axelrod (24) hold that glucuronic acid in the form of UDPGA conjugates with an acceptor molecule in the presence of an enzyme (which does not resemble β-glucuronidase in its properties) from cell-free extracts of rat liver. On the other hand, Fishman and Green (25) contend that β-glucuronidase catalyses efficiently the transfer of glucuronic acid from a conjugate to a suitable acceptor molecule. Since sufficient experimental evidence is available to support both the postulates, it was considered of interest to assay the liver samples of the three groups of rats for β-glucuronidase activity and the results are summarized in Table 5. The increase in the β-glucuronidase activity in the livers of hyperthyroid rats when considered with the observed decrease in the glucosiduronic acid synthesis by liver slices of hyperthyroid rats, clearly shows that *in this tissue at least,* β-glucuronidase may not be associated with glucosiduronic acid synthesis. The opposite holds true for hypothyroidal rats. Since the physiological function of β-glucuronidase apart from its being associated with the hydrolysis of glucuronides, is not well understood, it is not possible at this stage to offer any satisfactory explanation for the increase or decrease in β-glucuronidase activity observed in positive and negative thyroid stress respectively.

It is clear from the results presented in Table 2 that the overall detoxica-

tion of benzoic acid is much more efficient in hypothyroidal condition than in normal, whereas it is poor in hyperthyroidal condition. In the light of the results of the present investigation, it would be of interest to study the influence of thyroid stress on the detoxication of organic compounds like phenols, naphthalenes etc.

Acknowledgment

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THYROID FUNCTION IN EXPERIMENTAL NEPHROTIC SYNDROME[1]

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ABSTRACT

Thyroid function and thyroid hormone metabolism have been investigated in the experimental nephrotic syndrome produced by anti-kidney antibodies. Nephrotic animals differed from normal in three ways: (1) thyroxine was lost in the urine in conjunction with protein, to the extent of $11 \pm 7\%$ of a tracer dose; (2) the response to pharmacological doses of thyroxine, as determined by increase in oxygen consumption rate, was decreased by about 30%; (3) the rates of iodine uptake and of thyroxine secretion by the thyroid were increased, the latter by at least 40%. Since the response of the oxygen consumption rate to triiodothyronine was also decreased by about 30% even though this substance was not excreted to a significant extent in the urine, it was concluded that the first two changes represented primary abnormalities in iodine metabolism resulting from the nephrotic state and that the last was a compensatory change.

THE occurrence of lowered basal metabolic rate and elevated serum cholesterol in patients with the nephrotic syndrome led to the suggestion that such patients have a metabolic disturbance allied to hypothyroidism (1). This view received support from the later observation of diminished serum protein-bound iodine concentration in nephrotic patients (2). Recent studies have indicated that thyroidal uptake of iodine is in fact normal or increased in this condition, but there have been conflicting reports about the state of thyroxine metabolism (3, 4). Since some of the latter discrepancies may have been due to variability among the subjects with regard to degree of proteinuria, edema and iodine intake, the problem of thyroid-nephrosis interrelationships has been studied in the rat made nephrotic with anti-kidney serum. Several weeks after injection of serum, such animals have proteinuria, hypoalbuminemia and hyperlipemia, but no edema.

METHODS

Adult male rats maintained under constant environmental conditions (77–79° F and 40–42% relative humidity with constant lighting from 8 A.M. to 8 P.M.) were employed for all studies. Three types of diet were used: (a) Purina fox chow, (b) synthetic stock diet, high in iodine, (c) synthetic low-iodine diet of the same composition as (b) but

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having an iodine content of approximately 140 μg./kg. The synthetic diets were given by gavage or by *ad libitum* feeding; the former method provided a daily iodine intake of about 3 μg. in the case of the low-iodine diet.

Thyroidal radioactivity was calculated from the epithyroid count after correction for extrathyroidal neck activity (5). Urine was assayed in a scintillation well counter for total and protein-bound (insoluble in 10% trichloroacetic acid) radioactivity. Oxygen consumption was measured over fifteen-minute periods in an apparatus as described by Holtkamp *et al.* (6). Paper strip electrophoresis was carried out in veronal buffer at pH 8.6, ionic strength 0.075, at 120 volts for 15 hours. For localization of radioactivity, the paper strips were stained with bromphenol blue, scanned and then cut into segments 3 mm. wide. Each segment was counted in a scintillation well counter, and from the results a curve drawn of the distribution of radioactivity.

Iodine determinations were performed on thyroid glands and serum by the method of Zak *et al.* (7).

Nephrosis was induced by the method of Heymann and Lund (8) 3–4 weeks before the experimental period was started.

RESULTS

Studies of Thyroid Accumulation and Release of I^{131}

Accumulation gradient and thyroidal uptake. Five nephrotic and six control animals previously maintained on a low-iodine diet fed by gavage for 10 days were given 10 μc. of carrier-free NaI^{131} intraperitoneally; epithyroid counting was then performed at hourly intervals for 4 hours, and the accumulation gradients calculated (9). A further count was taken 18 hours after administration of the radioiodine. The results (Table 1) indicate a more rapid accumulation in the nephrotic rats, and a greater uptake at 18 hours.

Thyroidal release and urinary excretion of I^{131}. As a continuation of the above experiment, epithyroid counting was performed at 24-hour intervals, beginning 48 hours after injection of radioiodine. The negative of the

TABLE 1. THYROIDAL ACCUMULATION AND RELEASE OF RADIOIODINE IN NEPHROSIS

	Diet	Control	Nephrotic	P
Accumulation Gradient	Gavage	1.5 ± 0.1 (6)	3.3 ± 0.6 (5)	< .05
Uptake (%)	Gavage	15 ± 2 (6)	32 ± 3 (5)	< .01
	Ad lib.	37 ± 3 (6)	43 ± 2 (6)	< .05
Iodine Release Rate constant	Gavage	0.0338 ± 0.007 (6)	0.0579 ± 0.002 (5)	< .05
	Ad lib.	0.0659 ± 0.016 (6)	0.0863 ± 0.005 (7)	< .05
Per cent of dose 48–144 hours	Gavage		9.9 ± 1.3 (5)	
48–120 hours	Ad lib.		8.6 ± 0.7 (7)	
Thyroidal I^{127} (μg.) at midpoint of experimental period	Gavage	9.0 ± 0.8 (6)	7.5 ± 0.6 (5)	< .01
	Ad lib.	9.5 ± 0.4 (5)	10.0 ± 1.4 (5)	> .05

In this and all subsequent tables, the values shown are Mean ± Standard Error, while the number in parentheses indicates the number of animals used.

regression coefficient of logarithm of thyroidal radioactivity on time was taken as the rate constant of release of hormonal radioiodine. Radioactivity in urine was measured daily and fecal radioactivity was measured on pooled feces for the whole experimental period, for each animal. Immediately after the last neck count, serum concentration of I^{131} and I^{127} and thyroidal I^{131} were measured. Separate groups of normal and nephrotic animals treated in an identical manner were sacrificed at the midpoint of the experimental period and the thyroids analyzed for I^{127}; these values were taken as approximations of the mean thyroidal I^{127} for the experimental period in the main groups. A similar experiment was performed on six normal and seven nephrotic rats, maintained on a low iodine diet fed *ad libitum* for 5 days prior to injection of radioiodine; serum PBI^{131} measurements, however, were not made.

TABLE 2. SERUM PBI AND PBI^{131} IN NEPHROSIS

Experiment		Control	Nephrotic	P
1	PBI^{127} μg./100 ml.	3.0 ± 0.5 (6)	2.3 ± 0.3 (5)	$> .05$
	PBI^{131} % of dose/100 ml.*	3.6 ± 0.9	5.4 ± 0.8	$< .05$
2	PBI^{127} μg./100 ml.	4.9 ± 0.3 (6)	4.5 ± 0.4 (7)	$> .05$

* 144 hours after administration of I^{131}.

In both experiments the release rate constant was higher in the nephrotics (Table 1). The value of (rate constant × thyroidal iodine content) may be taken as an index of the rate of hormone secretion (as expressed for example in micrograms/day). The mean indices so calculated are .304 for control animals and .434 for nephrotics, indicating a mean increase in hormonal secretion in the latter group of about 41%. In the second experiment the mean increase was of the order of 37%.

By comparison with a standard, thyroidal radioactivity measured *in vitro* was calculated as a percentage of the administered dose. From the relationship between the *in vitro* and final *in vivo* counts, release of I^{131} as a percentage of the dose was calculated for the period beginning 48 hours after radioiodine administration (Table 1).

Serum PBI^{127} values were not significantly different in the two groups, but PBI^{131} concentrations were considerably higher in the nephrotics (Table 2). The mean relative specific activities of serum PBI, calculated from these measurements, were $1.23 \pm .18\%$ of dose per microgram for control animals, and $2.82 \pm .53\%$ per microgram for nephrotics.

The urinary radioactivity is shown (Table 3) for the period beginning 48 hours after administration of I^{131}; it may reasonably be assumed that all inorganic I^{131} excreted after this time was derived from degradation of

TABLE 3. URINARY AND FECAL EXCRETION OF I^{131} AFTER ADMINISTRATION OF RADIOIODINE (% of Dose)

Experiment			Total	Organic	Inorganic*
1 (48–144 hours)	Control (6)	Urine	5.8 ±0.5	0.06 ±0.01	5.7
		Feces		5.3 ±1.3	
	Nephrotic (5)	Urine	11.3 ±1.4	2.1 ±0.8	9.2
		Feces		6.1 ±1.4	
2 (48–120 hours)	Control (6)	Urine	7.6 ±0.6	0.045 ±0.01	7.6
		Feces		7.9 ±2.2	
	Nephrotic (7)	Urine	9.1 ±0.4	2.1 ±0.2	7.0
		Feces		9.0 ±1.1	

* By difference.

labelled hormone. Urinary excretion of organic I^{131} by nephrotic animals was considerably greater than normal. In the two experiments it amounted on the average to 19 ±6 and 25 ±4% of the radioiodine released from the thyroid gland, and to 18% and 23% of the total urinary I^{131} (calculated from data in Table 3). There was little PBI^{131} excretion until the proteinuria reached a level of about 300 mg. per day (Fig. 1); as protein excretion increased beyond this level, there was a progressive increase in the excretion of protein-bound I^{131}. The radioiodine content of feces pooled for the entire experimental period for each animal was not significantly different in the two groups.

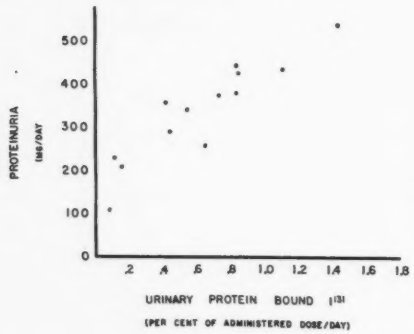


FIG. 1. Relationship between urinary protein and urinary PBI^{131}. Values are the means for the interval 48–120 hours after administration of inorganic I^{131}.

Metabolism of Thyroxine and Triiodothyronine

Control and nephrotic rats were given 0.5 μg. of *l*-thyroxine-I^{131} intravenously and the daily urinary excretion of I^{131} was measured for the next 3 days. Before and during this experiment the animals received a high iodine diet on an *ad libitum* basis to reduce thyroid uptake of I^{131} released

by degradation of thyroxine. It may be assumed that by the end of the experimental period virtually all I^{131} had been excreted in the urine or feces (10). The total urinary radioactivity of the two groups did not differ significantly, but the nephrotics excreted much more in a protein-bound form. Twenty-two per cent of the total urinary I^{131} in this group was protein-bound, a figure in good agreement with that of the preceding experiment in which the thyroxine had been endogenously labelled. The total PBI^{131} excreted in 3 days was $11 \pm 7\%$. The rate of thyroxine-I^{131} excretion was determined for each animal by extrapolating to zero time a semilogarithmic plot of urinary PBI^{131} (as percentage of dose) on time (days after thyroxine administration). The mean value obtained was $19 \pm 3\%$ of the dose per day. The relationship between protein and thyroxine excretion in the urine was similar to that previously observed after giving NaI^{131} (Fig. 2).

After administration of 0.07 μg. of *l*-triiodothyronine-I^{131} to four nephrotic rats, and measurement of urinary radioactivity for the next 2 days, it was found that $44.9 \pm 2.1\%$ of the administered radioactivity appeared in the urine but only $1.0 \pm 0.2\%$ of the dose was in protein-bound form. These values are comparable to those obtained in normal rats (20).

When blood and urine were examined electrophoretically after intravenous injection of 0.5 μg. of *l*-thyroxine-I^{131}, the radioactivity in serum was

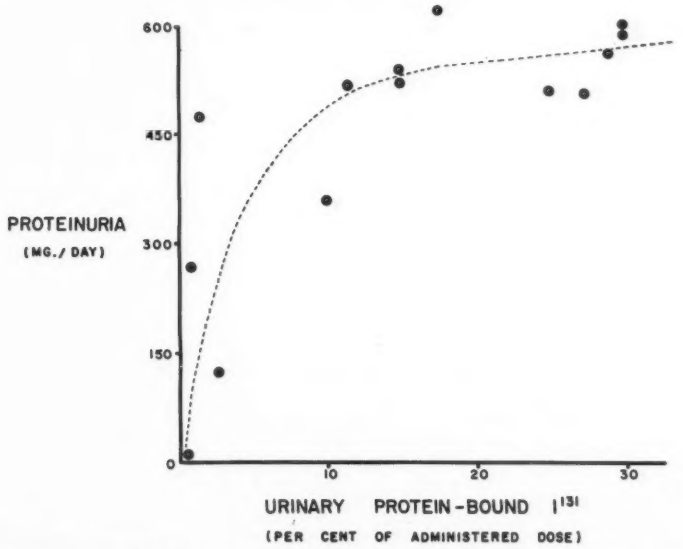


FIG. 2. Relationship between urinary protein and urinary PBI^{131} after administration of *l*-thyroxine-I^{131}. Values for protein are means for the first three days after injection of thyroxine. The values for PBI^{131} represent the rates of excretions in % of dose/day, calculated as described in the text.

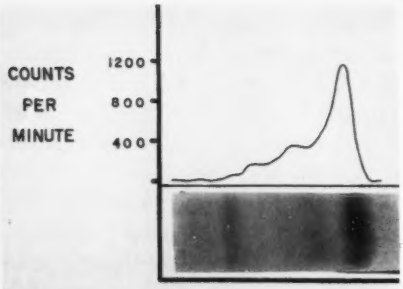


FIG. 3. Distribution of radioactivity of *l*-thyroxine-I^{131} in normal rat serum after electrophoresis.

distributed in two main peaks; one corresponding in mobility to the inter-alpha area, and the other to α_1 globulin or the area between α_1 and albumin. There was a small peak in the β globulin area as well. This pattern was qualitatively similar to that obtained in normal animals. The pattern in the urine differed only in that the front-running peak of radioactivity clearly coincided with the albumin peak (Figs. 3 and 4). Addition of thyroxine-I^{131} to nephrotic urine *in vitro* gave patterns identical to those obtained *in vivo*.

On electrophoretic examination of the serum and urine obtained 30 minutes after injection of 0.07 μg. *l*-triiodothyronine-I^{131} the radioactivity had the expected distribution in serum (11), showing association with the α globulins and extensive trailing in the regions of β and γ globulins; in the urine, however, it was associated largely with albumin.

Effect of Thyroid Hormones on Oxygen Consumption Rate

Oxygen consumption rates were determined before and during the administration of increasing doses of *l*-thyroxine and *l*-triiodothyronine,

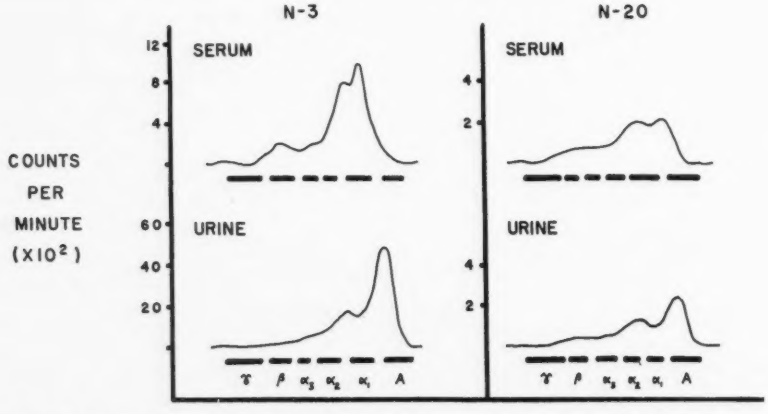


FIG. 4. Distribution of radioactivity in serum and urine of nephrotic rats after injection of *l*-thyroxine-I^{131}.

TABLE 4. URINARY EXCRETION OF I^{131} IN 3 DAYS AFTER ADMINISTRATION OF THYROXINE-I^{131}

	Per cent of Administered Dose	
	Total	Organic
Control (6)	56 ± 5	—
Nephrotic (13)	49 ± 21	11 ± 7

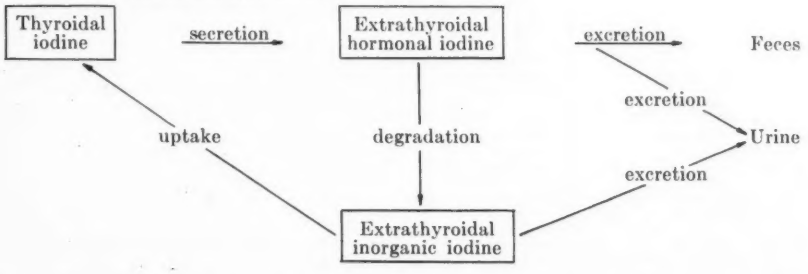
measurements being made 5–7 days after each increase in dose. The initial rates were 7.0 ± 0.1 liters/M^2/hour for the control and 7.2 ± 0.1 liters/M^2/hour for the nephrotics. Exogenous hormones were less effective in increasing the oxygen consumption in nephrotic animals by approximately 30% (Fig. 5).

Effects of Thyroxine on Serum and Urine Constituents in Nephrotics

The effects of thyroidectomy and of *l*-thyroxine replacement therapy were studied in nephrotic and control rats maintained on a constant food intake administered by gavage. The results (Fig. 6 and Table 5) in the two groups are qualitatively similar with regard to changes in body weight, urinary nitrogen and serum cholesterol, but quantitative changes in the last parameter were greater in nephrotic than in control rats. Plasma total protein concentration in both groups rose considerably following thyroidectomy, due to increases in serum globulin, especially α_1; there was a variable effect on the albumin concentration. Administration of 6 μg. of thyroxine daily by subcutaneous injection resulted in incomplete reversion to pre-thyroidectomy levels.

DISCUSSION

A simple model of iodine metabolism may be assumed as the basis for comparison of nephrotic and normal rats:



It was seen that the uptake of inorganic radioiodine and the rate of release of thyroidal radioiodine were increased in nephrotic animals. It was previously calculated that the nephrotics had a small but definite increase in the rate of hormone release (in micrograms per day), of the order of 37–41%, that the urinary excretion of PBI^{131} accounted for 18–23% of the

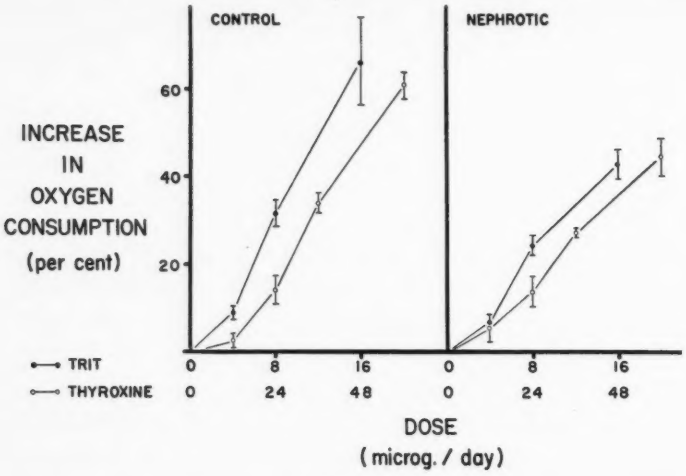


FIG. 5. Effect of *l*-thyroxine and *l*-triiodothyronine on oxygen consumption rates in control and nephrotic rats. Each point is based on 12–45 fifteen-minute periods; vertical bars represent S.E.M.

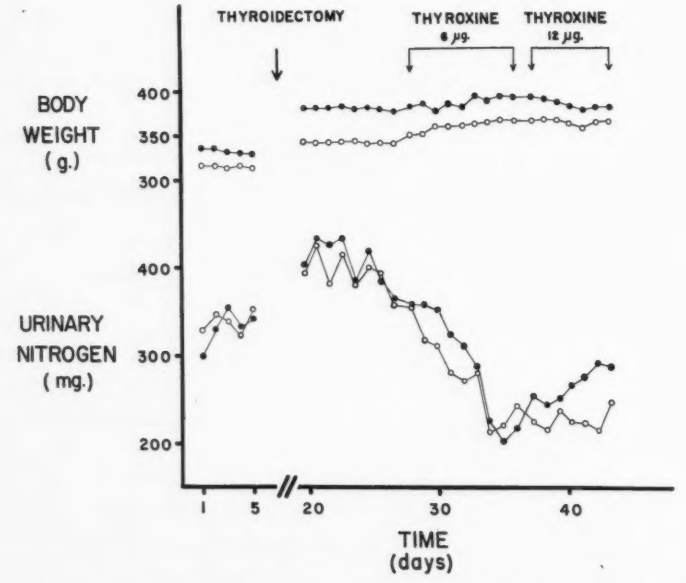


FIG. 6. Effects of thyroidectomy and *l*-thyroxine replacement therapy on body weight and urinary nitrogen excretion in control (•—•) and nephrotic (o—o) rats. Measurements were made daily. Values are means for 6 animals in each group.

TABLE 5. EFFECT OF THYROIDECTOMY AND OF THYROXINE REPLACEMENT ON SERUM CHOLESTEROL AND TOTAL PROTEIN CONCENTRATIONS

	Pre-thyroidectomy	Thyroidectomy	Thyroidectomy +Thyroxine (6 μg./day)
Cholesterol Change (mg./100 ml.)			
Control (6)		+ 61 ± 9	− 25 ± 10
Nephrotic (6)		+100 ± 38	−118 ± 15
Protein (gm./100 ml.)			
Control (6)	7.0	8.9	8.8
Nephrotic (6)	6.2	7.9	8.2

total urinary I^{131}, and that the urinary excretion of PBI^{131} accounted for 19–25% of the radioiodine released from the thyroid gland. The first and last of these figures are, however, minimum and maximum approximations, respectively, since they are based on the net release of I^{131} from the gland. It was unfortunately not feasible to block thyroidal reaccumulation of I^{131} by propylthiouracil, which in the usual blocking dose was lethal for nephrotic rats. Thus the actual release rate was higher and the proportion of secreted PBI^{131} lost in the urine lower than the values calculated. It therefore appears that the increased activity of the thyroid gland could be only partly the result of urinary loss of hormone.

When tube-fed, both normal and nephrotic animals showed a decrease in I^{131} uptake and I^{131} release rates compared to animals fed *ad libitum;* this is in agreement with recent work of Cohn *et al.* (12). It is of interest, however, that the difference between normal and nephrotic animals was still apparent.

The relative specific activity of serum PBI in the nephrotics was ap-

TABLE 6. EFFECT OF THYROIDECTOMY AND THYROXINE REPLACEMENT ON ELECTROPHORETIC PATTERN OF SERUM PROTEINS IN NEPHROSIS

Animal		Alb.	α_1	α_2	α_3	β	γ
Nephrotic	1	2.18	1.88	.89	.31	.73	.63
		2.05	2.28	.59	.89	1.09	1.22
		2.70	1.38	.68	.55	1.17	1.63
	2	1.89	2.16	.54	.41	.67	.41
		1.48	2.74	.84	.68	1.31	1.05
		2.22	2.61	.75	.50	.86	.89
	3	1.06	1.84	.45	.28	.45	.45
		1.17	3.59	.72	.63	.63	.81
		1.55	3.20	1.13	.83	.77	1.03
Control	1	3.50	1.30	.50	.50	1.00	1.10
		3.50	1.50	.50	.60	1.00	1.30
		3.80	1.20	.40	.30	1.40	1.10
	2	3.20	1.30	.40	.40	1.00	1.00
		4.10	1.70	.50	.50	1.80	1.20
		3.50	1.40	.70	.60	1.60	1.50

For each animal values shown are control (top line), thyroidectomy (middle line) and replacement therapy with 6 μg. of *l*-thyroxine daily (bottom line).

proximately twice as great as that of the normals; this is sufficient to explain the increased excretion by the nephrotics of inorganic I^{131} since this was derived from thyroxine degradation.

When labelled thyroxine was injected, the total amounts of I^{131} excreted by the control and nephrotic animals were not significantly different. However, in the latter group a considerable amount of this was lost as thyroxine and consequently not biologically effective.

The degree of such loss varied with the degree of proteinuria. The relationship between the two parameters, as observed in the rat (Figs. 2 and 3), may be explained (a) by a distinct T_M for the thyroxine-binding protein, or (b) by the assumption that the thyroxine-binding globulin is a large or asymmetrical molecule which does not pass the glomerular filter until this is severely damaged, as reflected in proteinuria of 200–300 mg. daily.

The electrophoretic studies were performed by conventional horizontal technique and therefore may suffer from certain artifacts of quantitation in the distribution of the thyroxine (13). However, for comparative purposes the results are valid and indicate, in contrast to the findings of Robbins *et al.* (14), that in the normal rat thyroxine is carried as in man (11, 15, 16), in association with a thyroxine-binding α globulin and with albumin. A similar pattern was obtained in the serum and urine of nephrotic rats.

The oxygen consumption studies indicate a normal rate of oxygen consumption by nephrotic animals. However, responsiveness to thyroxine was decreased by approximately 30%; this might be partly but not entirely attributable to urinary loss of the injected hormone. On the other hand, there was a similar decrease in response to triiodothyronine, yet urinary loss of this material was negligible. The effects of thyroidectomy and thyroxine replacement therapy on nitrogen excretion, serum globulins, serum cholesterol and body weight were qualitatively similar in normal and nephrotic rats, and were similar to those previously described (17, 18, 19). The greater effect of treatment on cholesterolemia in nephrosis is difficult to interpret because of the pre-existing disturbance in cholesterol metabolism.

The findings presented above indicate that in experimental nephrosis a considerable amount of thyroxine may be lost in association with protein in the urine and that there is a decreased responsiveness to administered thyroid hormones which can be attributed only in part to this urinary loss. These abnormalities are apparently compensated by an increase in thyroid activity; the adequacy of compensation is shown by the serum PBI levels and oxygen consumption rates, which under "normal" conditions were equal to those of control animals. The decreased peripheral response to thyroid hormones was apparently unrelated to any gross change in the mechanism of hormone transport.

The present results are compatible with those of Rasmussen (4), obtained in nephrotic patients, indicating that urinary excretion may account for a significant fraction of the thyroxine entering the circulation. If the observed relationship between urinary thyroxine loss and proteinuria applies to the human, disagreement (3, 4) concerning the extent of thyroxine loss may be related to only small differences in degree of proteinuria in the subjects studied.

The relationship of experimental nephrosis to the nephrotic syndrome in humans is far from clear. The gross manifestations are identical, probably because these are consequent on massive proteinuria in both cases. In the present work the major difference between the two states is the apparent inability of the human to compensate for the two abnormalities in thyroxine metabolism, so that the BMR and serum PBI remain low. Rasmussen (4) has postulated that this is due to a degree of hypopituitarism secondary to protein deficiency. That this apparently did not obtain in the nephrotic rat may be a manifestation of differences in the metabolic channeling of available protein rather than of a fundamental difference in thyroid function and iodine metabolism.

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THYROID EPITHELIUM IN TISSUE CULTURES[1]: OBSERVATIONS ON THE MORPHOLOGY AND FUNCTIONAL CAPACITIES OF EMBRYO CHICK THYROIDS

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ABSTRACT

Chick embryo thyroid fragments were cultivated in special tissue culture chambers designed primarily for cytological investigations of the living cell. Employing time-lapse techniques three secretory phenomena were observed. These were associated with (1) cytoplasmic droplets which were equivocally PAS positive and (2) cytoplasmic phase contrast green granules, the localization sites for I^{131}. Chicken whole egg ultrafiltrates stimulated the epithelial outgrowths to a marked degree but this was not apparent for the fibroblastoid stromal elements.

IN 1911 Carrel and Burrows (1) described tissue culture outgrowth of thyroid glands from dogs, cats and guinea pigs predominately as fibroblastoid with occasional epithelial sheet emigrations. Since their presentation four reports (2–4 and 23) have indicated the occurrence of epithelial outgrowths from thyroid tissue cultures but the bulk of analyses (5–21) have been limited to studies of whole gland sections after *in vitro* cultivation. Previous reports indicated that tissue cultures of chick embryo (3) and young adult rabbit (4) epithelium were not functioning. This report introduces data using other tissue culture techniques which demonstrate a functional capacity of chick embryo epithelium.

MATERIALS AND METHODS

(1) *Thyroid source.* Fertile eggs of a hybrid strain (New Hampshire reds×white leghorns) were used as outlined in previous reports (29 and 30) at eight and one-half days and at thirteen days of incubation. Thyroid glands were removed with fine forceps and immediately immersed in *Medium No. 1036* (a variation of Medium No. 858 (31)), trimmed of as much connective tissue as possible and then divided with scalpels.

(2) *Cultivation.* Three or four pieces of divided thyroids were placed on a sterile No. 1 (43×50 mm.) coverslip in a drop of heparinized cockerel plasma. The embryonic thyroid alone produced a firm clotting of the plasma so that additional embryonic extract

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was not necessary. The rubber gaskets and opposing coverslips were then positioned and the multipurpose culture chambers (24–27) closed. Eight to twenty-four chambers were set up in this way for each experiment. Generally, a half or a third of the chambers were selected at random as the control and these were filled with 2 ml. of *Medium No. 1066*. The experimental groups were filled with *Medium No. 1036* which contained varying percentages of chicken whole egg ultrafiltrate (28). Fluids were exchanged after the first two or three days and after five or six days.

(3) *Phase contrast observations and recording.* Each explant was examined with the phase contrast microscope for total growth and epithelial sheet emigration. Epithelial activity was recorded by the time-lapse camera on color and black and white movie film as it was observed through AO-Baker interference and *B&L* phase contrast microscopes (22 and 23).

(4) *PAS staining.* Colloid formation was ascertained by *PAS* staining according to modified techniques of Hotchkiss (32) and others.

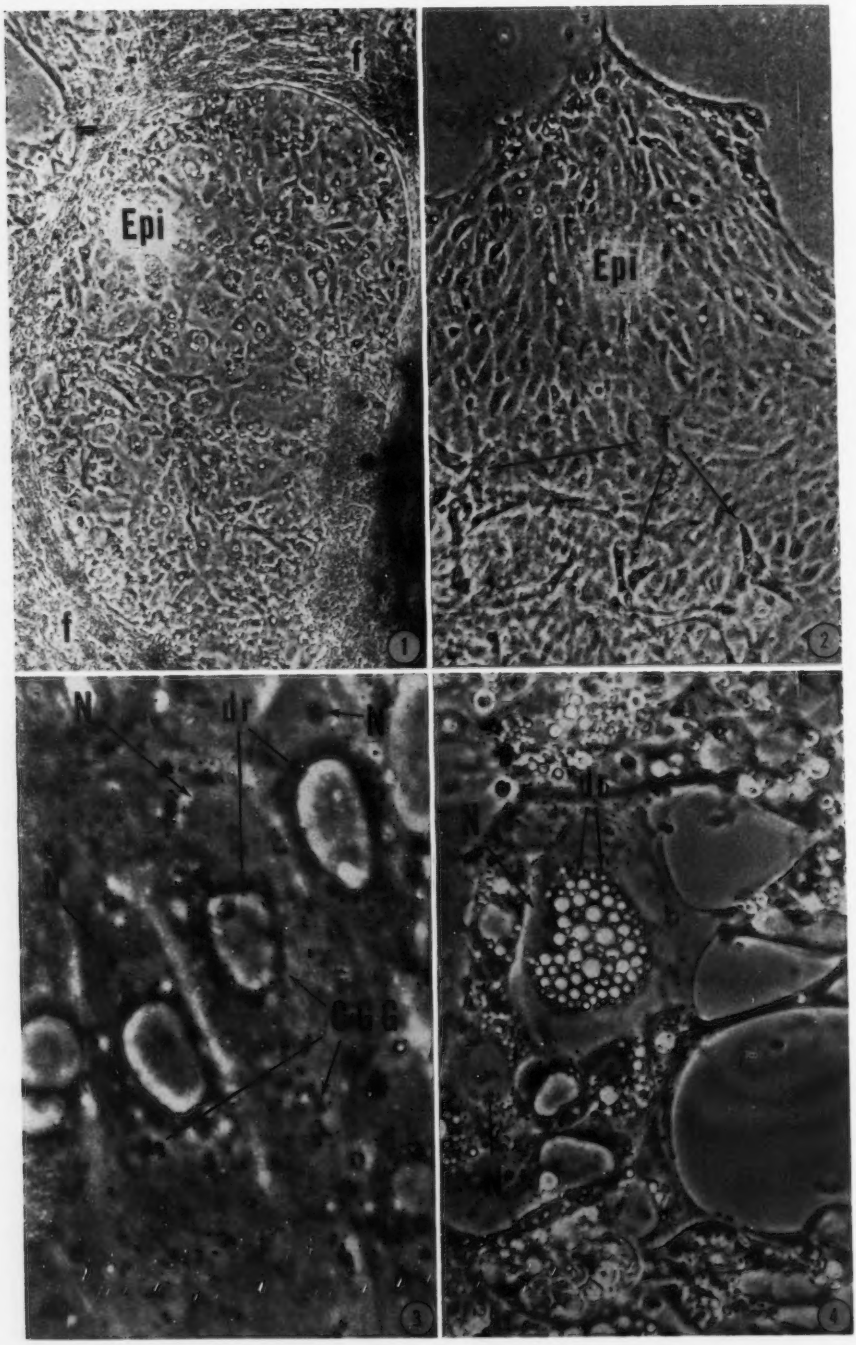
(5) *Radioactive iodine* (I^{131}). Explants cultivated five to six days were exposed for a 24-hour period to nutrients which contained in addition to their control or experimental ingredients I^{131} (1 μ curie/ml.). After this exposure the cultures were fixed in formol, washed in water and covered with stripping film (Kodak) in a manner outlined elsewhere (33).

(6) *Thyroid Stimulating Hormone* (*TSH*). A commercial preparation of the Armour Laboratories (*Thytropar*) was used as the thyroid stimulating hormone in these studies. This lyophilized product was diluted with appropriate amounts of *Medium No. 1063* just prior to its introduction into the chambers so that the cultures received 0.5 unit (U.S.P.) per one ml.

RESULTS

The following general description of development of these thyroid fragments within the culture chambers applies to both the $8\frac{1}{2}$- and 13-day embryo thyroids.

Phase contrast observations. After one day of cultivation a few cells of unclassified origin emigrated around the peripheral edges of the explants. Adjacent to many explants there was a circular or oval lysed area in the clot. In some cases, by the second day, spindle cells emigrated around the peripheral edge of this lysed portion of the clots which had generally enlarged since the first day and syncytia grew on the glass into these clear areas. In other instances, syncytial sheets grew outside of the lysed area and directly upon the surface of the clot. After five or six days of cultivation the epithelial cells had definite membrane borders and the amount of fibroblastoid outgrowth varied widely among the explants (Figs. 1 and 2). Large cytoplasmic droplets occurred within the epithelial cells (Fig. 3), notably in those forming the periphery of the sheets. Generally, these droplets were singular but occasionally cells were noted with many lesser-sized droplets (Fig. 4). Mitotic divisions were rarely observed in these epithelial sheets. The nuclei were regular and small (8–10 μ) and generally had one prominent nucleolus. The cytoplasm contained varying amounts of coarse granules and these too occurred more abundantly in cells at the periphery of the epithelial sheets (Fig. 2). Some of these granules lay adjacent to and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on the surface of the large cytoplasmic droplets (Fig. 3). At the borders of the epithelial sheets there were points at which the cells were denser, rounder and more granular. Many of these cells also contained the cytoplasmic droplets and several large coarse granules. Careful observation revealed that many of the cytoplasmic granules appeared green regardless of their size when viewed with the phase contrast system (B&L). With bright field optics they were faintly yellow.

Time-lapse motion picture studies. This method of analysis was especially useful for studying epithelial activity. The large cytoplasmic droplets were observed in an effort to determine whether they were involved in a secretory phenomenon. Cells possessing these droplets moved slowly to the edges of the epithelial sheets and there in an un-elicited manner the droplets disappeared as the cells became spheroidal, granular and detached from the epithelium. Some of these detached cells passed into the fluid medium (Figs. 5–9). The fluid of cytoplasmic droplets in other cells was rapidly eliminated (one or two minutes) by a rupture of the droplet wall (Figs. 10–14). A third activity involving a fluid droplet occurred as a rather violent cellular activity which resulted in a protoplasmic flow through a ruptured cell wall. In doing this, the droplet also was lost from the cell (Figs. 15–29).

PAS staining. The explants of cultures subjected to this technique appeared a deep magenta. In some cases the cytoplasmic droplets demonstrated a positive PAS stain but the density of their coloration was light as compared to those of the explants. The phase contrast green granules previously mentioned which are also those cited with reference to radioactive iodine were not involved in this *PAS* staining. Cells which had migrated to the periphery of the epithelial sheets and had rounded as previously described were *PAS* positive even after diastase digestion.

Radioactive iodine (I^{131}). Cultures which were subjected to radioactive iodine *in vitro* and prepared for stripping film analysis demonstrated that

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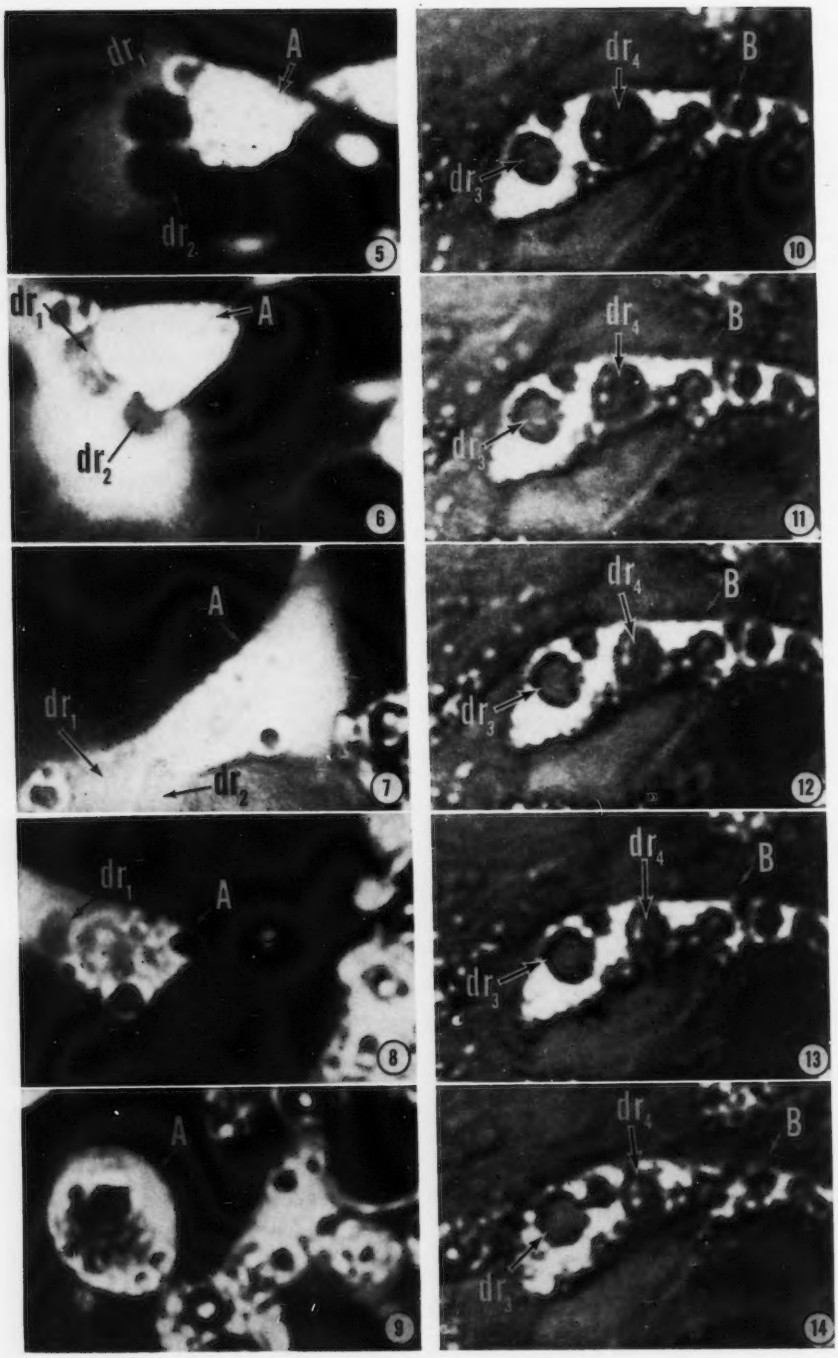
FIGS. 1–4. Thirteen-day embryo chick thyroid epithelium growing on glass slides in multipurpose culture chambers for five to seven days as observed through the phase contrast microscope.

FIG. 1. Epithelial sheet (Epi) surrounded by a fibroblastoid stroma (f). ×125.

FIG. 2. Periphery of epithelial sheet with coarse green granules at its limiting edge, cytoplasmic droplets scattered throughout and encroaching fibroblastoid cells (f). ×125.

FIG. 3. Oil immersion (1.8 mm.) view of several epithelial cells which possess large cytoplasmic droplets (dr) and coarse green granules (cgg) scattered throughout the cytoplasm and in close proximity with the cytoplasmic droplets. Note the adjacent location of the nuclei (N) to the three largest cytoplasmic droplets. ×1200.

FIG. 4. Here is a less common occurring multiplicity of cytoplasmic droplets about the nucleus. This formation was seen most commonly in cultures containing serum supplements and was not observed in epithelialization such as demonstrated in Fig. 2. ×550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dr3
14

the coarse cytoplasmic granules (green with phase contrast) were the sites affected by the I^{131}. With the exception of adjacent coarse granules, the cytoplasmic droplets were not involved with the I^{131}. The greatest concentration of radioactivity occurred in cells at the periphery of the epithelial sheets. The adjacent spindle cells occasionally demonstrated an I^{131} concentration; however, explants from supportive tissues which showed only spindle cell outgrowth without clot lysis did not demonstrate this activity. Epithelium cultivated in *Medium No. 1066* with or without the whole egg ultrafiltrate demonstrated I^{131} uptake in a similar way.

Thyroid Stimulating Hormone (TSH). With the time-lapse movie equipment an epithelial cell was observed to develop a perinuclear cytoplasmic droplet after TSH (0.5 U.S.P. units/ml.) was introduced into the nutrient fluid. Following this development, the droplet suddenly disappeared by the method indicated previously (Figs. 10–14) in which the fluid droplet was lost to the nutrient medium by a rupture of its wall.

Growth vs. Chick Whole Egg Ultrafiltrate. Table 1 summarizes the effect on the total growth of explants and their capacity to proliferate epithelial sheets under the aegis of a control (*Medium No. 1066*) nutrient versus one which contained chicken whole egg ultrafiltrate. The top half of Table 1 summarizes the data of the $8\frac{1}{2}$-day chick embryo thyroids cultivated in a control nutrient and in one containing whole egg ultrafiltrate (5%) for 5.5 days. This series comprised a study which involved eight experiments and an analysis of 345 explants. Of the control group, 67% of the explants

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FIGS. 5–9. This series was taken from a 16 mm. time-lapse movie sequence and represents an analysis over a four-hour period of an 8.5-day chick-embryo thyroid after five days of cultivation (cell A). The series was originally recorded on Kodachrome "A" film as viewed through the AO-Baker interference microscope. ×1000.

FIG. 5. This cell has two cytoplasmic droplets, dr_1 and dr_2. The nuclear area is located to the right of these two droplets.

FIGS. 6–7. The cell is seen to be moving in a counterclockwise direction and one of the droplets, dr_2, was either released or combined with dr_1 after Fig. 7.

FIG. 8. Only one fluid droplet is now observable and from observations made of the projected sequence it appeared to be dr_1, possibly dr_1 plus dr_2.

FIG. 9. The cell in this illustration is now a globose mass detached from the epithelium of its origin and ultimately will drift into the fluid nutrient. This type of cell demonstrated the most marked uptake of I^{131}.

FIGS. 10–14. This series of an 8.5-day chick embryo thyroid cultivated for five days was similarly taken from a 16 mm. time-lapse movie originally made on Kodachrome "A" film and observed through the AO-Baker interference microscope. The sequence shows a pair of prominent cytoplasmic droplets (dr_3 and dr_4) contained within cell B. Several smaller fluid droplets are also noted. One droplet (dr_4) which is the most central and largest may be followed to rapidly diminish in size. In the movie projection this was visualized as a "popping" of the fluid droplet and rendered the impression that the fluid was suddenly released through the thin cellular wall into the fluid nutrient. The sequence time was four minutes. ×1000.

TABLE 1. GROWTH RESULTS AFTER 5.5 DAYS OF CULTIVATION OF 8.5-DAY CHICK EMBRYO THYROIDS (SUMMARY OF EIGHT EXPERIMENTS)

	Control (1066)			Experimental (1066-WE 5%)		
	(a)	(b)	(c)	(a)	(b)	(c)
Totals	178	119	36	167	128	63
Growth		67%			77%	
Epithelium		20%			38%	

GROWTH RESULTS AFTER 5 DAYS OF CULTIVATION OF 13-DAY CHICK EMBRYO THYROIDS (SUMMARY OF EIGHT EXPERIMENTS)

	Control (1066)			Experimental (1066-WE 5%)		
	(a)	(b)	(c)	(a)	(b)	(c)
Totals	201	180	45	245	232	188
Growth		90%			95%	
Epithelium		22%			77%	

Control (1006). Explants in plasma clots (no EE) and fluid nutrient composed of Medium #1066.

Experimental (1006-WE 5%). Explants in plasma clots (no EE) and fluid nutrient composed of Medium #1066 with whole egg ultrafiltrate (5%).

(a) Total number of explants for given experiment.

(b) Number of explants demonstrating any type of cellular emigration.

(c) Number of explants demonstrating epithelial sheet emigration.

Growth. Percentage of explants with emigrating cells of any type.

Epithelium. Percentage of explants with epithelial sheet emigration.

had outgrowths (from a few to an abundance of emigrating cells) whereas in the experimental group which contained the whole egg ultrafiltrate (5%) 77% of the explants demonstrated this type of outgrowth. In the control group, 20% of the explants had some degree of epithelial emigration whereas in the experimental group 38% of the explants had a similar emigration. This represented a 10% increase for the explants with outgrowths and an 18% increase for the explants with epithelial emigrations in the experimental group. The lower half of Table 1 represents a similar analysis of 13-day chick embryo thyroids after five days of cultivation. Both the control and experimental nutrients were identical to those for

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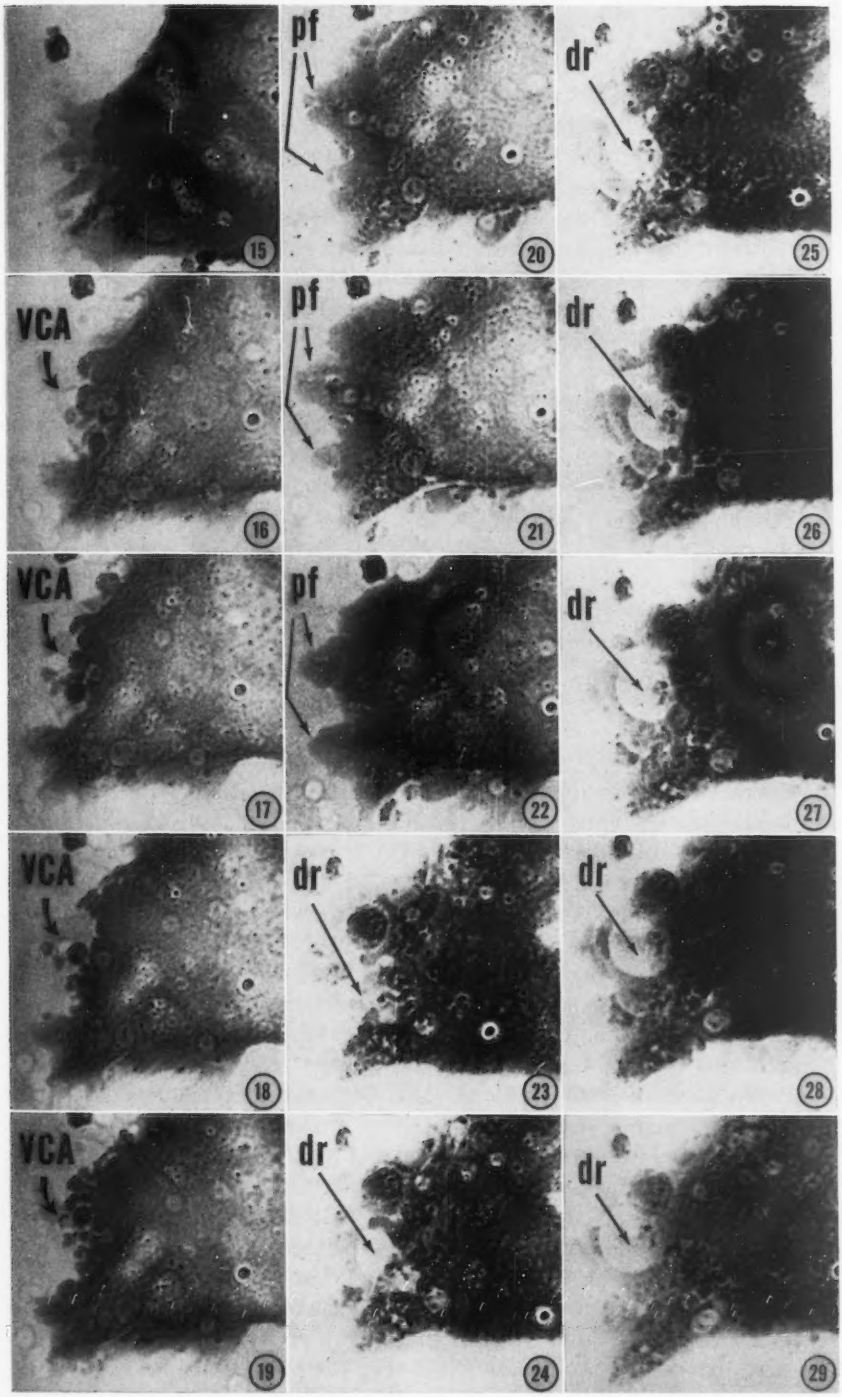
FIGS. 15–29. These pictures of an 8.5-day chick embryo thyroid cultivated for five days were taken from a 16 mm. time-lapse film originally taken on Kodachrome "A." They were observed through the AO-Baker interference microscope over a period of four hours and represent a recorded form of secretory activity. ×600.

FIG. 15. The cytoplasmic area before secretory activity was noted.

FIGS. 16–19. Violent cellular activity (VCA) which preceded the protoplasmic flow onto the glass substrate.

FIGS. 20–22. Here the protoplasmic flow (pf) is indicated to follow the violent cellular activity and occurred at the same position.

FIGS. 23–29. Pictures successively show the emergence of a single large fluid droplet (dr) from the interior of the cytoplasm to its most peripheral portion.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VCA
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dr
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the 8½-day thyroids. This study involved eight experiments and an analysis of 446 explants. In the control group 90% of the explants demonstrated some type of outgrowth and in the experimental group 95%. In the control group, 22% of the explants demonstrated an epithelial emigration and in the experimental group, 77%. The total outgrowth was then 5% greater in the experimental group which contained the whole egg ultrafiltrate (5%) and the epithelial emigration of the experimental group was 55% greater than the control group. A similar analysis of 13-day chick embryo thyroids in control and experimental groups which contained varying amounts of the whole egg ultrafiltrate (1, 2, 5 and 10%) was carried out in four smaller experiments. The outgrowths in every condition (controls and experimental groups) were in excess of 90% with the exception of the whole egg ultrafiltrate 2% which was recorded at 81%. The epithelial emigration for the control was 33% whereas for the whole egg ultrafiltrate groups it was in excess of 66% with a maximum of 75% for the whole egg ultrafiltrate (5%).

DISCUSSION

Primarily, this study has attempted to show that (1) the epithelial cells which emigrated from embryo chick thyroids were cells which retained at least a part of their functioning capacity and (2) chicken whole egg ultrafiltrate specifically assisted in the reduplication of these epithelial cells by the technique employed. We felt that the first point was a basic consideration which must be proved or disproved before the effect of certain agents incorporated into the nutrient media could be evaluated. If we could not establish that the epithelial emigration consisted of thyroid functioning cells, then any means by which we might stimulate the growth of such an epithelium would have less importance. The fact that the work of Gonzales (3) did not reveal *PAS* positive droplets within the epithelial sheets of chick embryo thyroids cultivated in roller tubes and the fact that Oppenheimer *et al.* (4) were unable to stimulate activity in the rabbit thyroid epithelium after TSH had been incorporated into the nutrient, nor to observe stripping film emulsion reduction after I^{131} had been incorporated into the nutrient did not deter pursuit of this basic information. In the work of Gonzales it seemed quite likely that the droplets of the epithelial sheets were so flat (less than 1 μ) compared to the droplets in the explants themselves that they appeared as a negative *PAS* reaction. When we observed epithelia which contained large cytoplasmic droplets, we considered this possibility and with very critical illumination were able to see these droplets in some cases as a fuchsia of positive *PAS* reaction. Some slides demonstrated this to a greater extent than others.

When 5- and 6-day-old cultures were influenced by nutrients which contained radioactive iodine (I^{131}), fixed and subjected to stripping film photographic techniques, it was noted that the phase contrast green cyto-

plasmic granules in the epithelial cells were the sites of radioactive uptake. It was felt that the green granules of the living and fixed cultures must have the same optical density and therefore, represented the same organoid. These granules (living or fixed preparations) were prominent in the peripheral cells, particularly in those which had rounded, and, therefore, have taken on a metabolic importance which demands further investigation of this type. Their increasing numbers in the peripherally located cells which are being shed from the sheets point to the functioning capacity of these tissue cultures (34).

In following these epithelial sheets with cinematography we were confronted by three potential means of cellular secretion. All of these methods were observed more than one time so it would be difficult to pass them off only as unusual events. All three phenomena involved the cytoplasmic droplets and, therefore, they were assumed to contain a secretory product. Since the droplets were equivocally *PAS* positive and did not take up the I^{131}, they were probably a colloid source, perhaps a precursor. The luminous-phase-contrast-green granules which did take up the I^{131} were probably associated with thyroxin production.

On several instances the thyroid stimulating hormone *Thytropar* (Armour) was introduced into tissue culture chambers which had been observed for a previous period. Although this was a tedious procedure involving cinematographic and still-camera time-lapse methods it was apparent that the cytoplasmic droplets did increase in numbers during the first twenty-four hours. This was not offered in the *Results* section because occasionally we saw an increase of cytoplasmic droplets occur in cultures which had not received this hormone.

In the analyses which involved thyroid growth in the presence of control (*Medium No. 1066*) and experimental (*Medium No. 1066* plus whole egg ultrafiltrate) nutrients, there appeared to be a consistent increased epithelial stimulation in the $8\frac{1}{2}$-day embryo thyroids as well as a concomitant but lesser increase in the total growth of these explants due to the whole egg ultrafiltrate. The increase did justify further evaluation of another age group (13-day chick embryo thyroid). Within this group we found an extreme epithelial surge which was not paralleled by the total explant outgrowth inasmuch as this was already at a high value (90%). There appeared to be a real need or a mechanism of utilization by this age explant for the whole egg ultrafiltrate as the epithelial response was more than three times greater than in thyroid explants cultivated in its absence. The analysis indicated, too, that even very small amounts of the whole egg ultrafiltrate affected epithelial proliferation. In analyzing the control groups of both $8\frac{1}{2}$- and 13-day chick embryo thyroids the epithelium was often just barely perceptible and in some cases it appeared to be damaged or waning and was not considered to appear as healthy as those cultivated in the whole egg ultrafiltrate. The figures then

are misleading with respect to the amounts of epithelium proliferated in the various conditions.

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A DIRECT INFLUENCE OF 5-HYDROXYTRYPTAMINE ON THE ADRENAL CORTEX[1]

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ABSTRACT

An *in vitro* comparison of adrenals treated with and without 5-hydroxytryptamine (serotonin) has permitted the demonstration that serotonin has a direct effect on the adrenal cortex. An increase in the secretion of blue tetrazolium (BT) reactive reducing material was elicited by serotonin from the adrenals of rabbits, guinea pig, rat and cow. The response was observable at serotonin levels between 100 to 1000 μg./pair of adrenals. Neither reserpine nor epinephrine had a comparable effect and no alterations in BT-titers were found in kidney and liver slices treated with serotonin. Although serotonin had a similar quantitative effect like ACTH on adrenal BT content, the serotonin influence could be distinguished by the appearance of non-α-ketolic reducing material. The nature of the increased BT material has been partially suggested by its chromatographic and biological behavior. On silica gel columns and toluene-propylene glycol chromatograms, the BT substance(s) migrated similarly to steroids. The material also exhibited sodium retention activity greater than deoxycorticosterone. The lability of the material has prevented identification while the non-α-ketolic reducing compounds have not been investigated at the moment. The possible biological importance of this adrenal reaction to serotonin was discussed.

IN A recent publication Moussatche and Pereiro (1) postulated the liberation of adrenocorticotropin (ACTH) by 5-hydroxytryptamine (serotonin). These investigators obtained a decrease in adrenal ascorbic acid after intravenous injection of serotonin into the intact but not the hypophysectomized rat. It was of interest to us to determine whether serotonin could elicit a direct effect upon the adrenal. By *in vitro* comparison of serotonin-treated and untreated preparations of adrenal tissue, it was possible to assign a stimulating activity of 5-hydroxytryptamine precisely to the adrenal cortex itself. In the presence of serotonin the content of the reducing substances associated with the steroid fraction of the adrenal was increased. Neither reserpine nor epinephrine could bring forth a comparable elaboration of blue tetrazolium reactive material.

METHODS AND EXPERIMENTAL

Adrenals from rabbits, guinea pigs or rats were dissected into quarters or sixths and were incubated for 2 hours in Krebs-Ringer phosphate buffer containing glucose (2, 3). Eight animals of each species were employed so that a representation of each adrenal

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[1] This work was supported by grant number A-1207 from the Public Health Service.

from each animal was present in all the Warburg flasks used in a particular experiment. Adrenal homogenates were also studied and these were prepared by grinding 8 pairs of adrenals in 25 ml. of medium in an all glass homogenizer for approximately 2 min. Debris was removed by filtration through cheesecloth. Adrenal cortical slices from the cow were also investigated.

When glands were relatively large, the incubation flasks contained 30 to 50 mg. (dry wt.) of tissue in 3 ml. of solution. Less available tissue (8 to 15 mg. dry wt.) as in the case of the rat adrenals was incubated in 2 ml. volumes. The compounds to be tested were tipped in from the side arm after the manometers were equilibrated. Astwood's oxycellulose purified ACTH was used as a standard to indicate the capacity of the adrenal to respond and was compared with 5-hydroxytryptamine (as the creatinine sulfate), reserpine and epinephrine. The gaseous phase was always 100% oxygen.

After incubation, the medium and a $\frac{1}{2}$ or 1 ml. rinsing of the flask were transferred to a 5 or 10 ml. glass stoppered tube containing an equal volume of specially purified methylene chloride (2) and shaken. Aliquots of the organic layer from a single extraction were subjected to analysis for reducing material. In the blue tetrazolium test (BT), the sample was dissolved in 0.5 ml. ethanol and 0.25 ml. of 0.018N tetramethylammonium hydroxide was introduced followed by 0.25 ml. of 0.1% blue tetrazolium in ethanol (3). An estimate of non-α-ketolic substances like tocopherols, vitamin K and 5-hydroxyindole derivatives that might be extracted into the methylene chloride was obtained by the ferric chloride-α,α'-dipyridyl test (EE)(3).[2] The sample was dissolved in 1.5 ml. ethanol and alternately mixed with 0.25 ml. of 0.4% α,α'-dipyridyl in absolute ethanol and 0.25 ml. of 0.2% ferric chloride in methanol. Readings at 520 mμ were taken 30 seconds after addition of the ferric chloride. Subtraction of EE values from BT levels would correct the steroid samples for possible non-steroid substances that give a positive BT test.

Attempts at isolation of the reducing material were carried out by incubating larger quantities of rabbit and rat adrenal tissue. Representative portions of each adrenal from 32 rats were incubated with and without serotonin under similar conditions used for the small scale studies. This was repeated using the whole or demedullated adrenals from groups of 12 rabbits. The pooled quartered glands served as their own controls; an initial $1\frac{3}{4}$ hour incubation contained no serotonin and was followed by an identical interval of time with fresh medium and serotonin. The corresponding methylene chloride extractions were taken down to dryness under vacuum and the residues were chromatographed. Pooled residues from extracts of adrenals from more than 100 rabbits were introduced onto a gm. 5 silica gel column which was eluted consecutively with 40 ml. volumes of benzene, benzene-methylene chloride mixtures, ethyl acetate and methanol. Pooled residues from extracts of adrenals from less than 30 rabbits were subjected to paper chromatography in toluene-propylene glycol or toluene-chloroform-ethylene glycol for 16 to 24 hours (4).

RESULTS

The results of these studies are given in Tables 1 and 2 with all determinations performed in duplicate. Neither creatinine nor sodium sulfate could elicit the response of serotonin creatinine sulfate. In Table 1 may be seen the influence of serotonin on quartered rabbit adrenal. A clear increase in blue tetrazolium reactive material has been elicited. Identical results were obtained with demedullated adrenal preparations. This increment in reducing substances becomes especially significant since the

[2] Rosenkrantz, H., *Arch. Biochem. & Biophys.*, in press.

TABLE 1. THE INFLUENCE OF 5-HYDROXYTRYPTAMINE ON THE CONTENT OF REDUCING MATERIAL IN THE QUARTERED RABBIT ADRENAL*

Experiment	Additive	Corrected BT†	EE	Change
		μg./100 mg. dry weight		%
1	0	76	0	
	ACTH‡	161	0	+110
	1 mg. 5-HTA§	127	97	+ 68
2	0	78	0	
	ACTH	121	0	+ 55
	0.5 mg. 5-HTA	117	27	+ 50
	1.0 mg. 5-HTA	122	53	+ 56
3	0	24	0	
	ACTH	43	0	+ 79
	0.5 mg. 5-HTA	41	28	+ 70
	1.0 mg. 5-HTA	75	48	+212
	1.0 mg. epinephrine	14	0	− 41
	0.5 mg. reserpine‖	20	14	− 16
4	0	45	0	
	1.0 mg. 5-HTA	123	15	+173
	1.0 mg. reserpine	53	5	+ 17

* The rabbits weighed approx. 2000 gm. and both sexes were used.

† EE and BT values are based on serotonin and cortisol standards. Subtraction of the EE titers from the BT level corrects for interference by serotonin metabolites that may have been extracted. Serotonin and α-ketolic steroids give approximately equimolar densities in the BT test.

‡ 8 units of Astwood's ACTH used.

§ 5-HTA = 5-hydroxytryptamine (serotonin) creatinine sulfate.

‖ Added as a suspension.

TABLE 2. THE INFLUENCE OF 5-HYDROXYTRYPTAMINE ON THE CONTENT OF REDUCING MATERIAL IN QUARTERED ADRENALS FROM OTHER SPECIES

Species	Additive	Corrected BT*	EE	Change
		μg./100 mg. dry weight		
Guinea pig	0	7	0	
(600 g., males)	ACTH	20	0	+185
	0.5 mg. 5-HTA	9	1	+ 22
	1.0 mg. 5-HTA	23	9	+100
	1.0 mg. reserpine	6	1	− 14
	1.0 mg. epinephrine	6	1	− 14
Rat	0	260	0	
(250 g., females)	ACTH	310	0	+ 19
	1.0 mg. 5-HTA	300	200	+ 15
Rat	0	171	0	
(250 g., males)	ACTH	200	32	+ 17
	1.0 mg. 5-HTA	210	231	+ 22
Rat†	0	200	0	
(250 g., males)	5-HTA	280	120	+ 40
Cow	0	26	0	
(slices)	ACTH	30	0	+ 15
	1.0 mg. 5-HTA	31	5	+ 19
	1.0 mg. epinephrine	21	0	− 19
	1.0 mg. reserpine	22	0	− 15

* EE and BT values are based on serotonin and cortisol standards. Subtraction of the EE titers from the BT level corrects for interference by serotonin metabolites that may have been extracted. Serotonin and α-ketolic steroids give approximately equimolar densities in the BT test.

† Determinations from a large scale incubation.

values of the non-α-ketolic compounds (those that react with ferric chloride-dipyridyl) have been subtracted from the total BT content. A plot of the elaboration of ferric chloride and blue tetrazolium reducing material, respectively, is given in Figure 1 for varying concentrations of serotonin. In the case of the EE curve, EE control levels were subtracted from each point. For the BT content both BT control levels and total EE titers were subtracted at each dose level of serotonin. It can be seen in

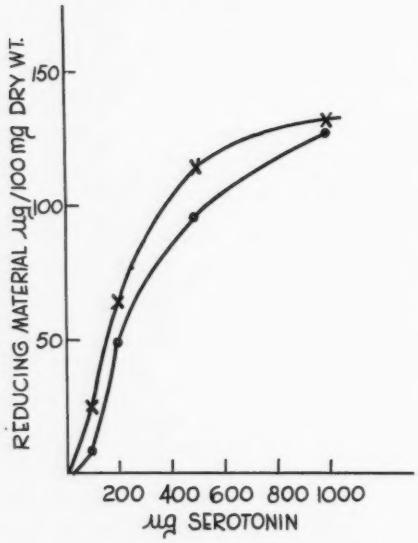

FIG. 1. The elaboration of reducing material from rabbit adrenals induced by serotonin: ×——× ferric chloride-dipyridyl reaction. ·——· blue tetrazolium levels.

Figure 1 that after a slight lag period that both EE and BT concentration of the incubation medium increase similarly and plateau at a serotonin level near 1000 μg.

That this is not a non-specific effect of serotonin is demonstrated by the lack of a comparable effect by epinephrine or reserpine (Table 1). The elevation of adrenal BT material is of the same order of magnitude as brought forth by ACTH stimulation. The mean percentage increase due to ACTH was 81 while that of serotonin was 105. Both substances required oxygen and glucose for maximum effect. It is realized that the dosages used for both ACTH and serotonin are much greater than those needed for *in vivo* stimulation. Kidney and liver slices were used as control tissues and serotonin did not alter their BT-titers.

A distinction between the ACTH and serotonin effects can readily be made on the basis of the EE reaction. Little or no material which gave a positive EE test was produced in the presence of ACTH while 5-hydroxytryptamine caused the release of significant quantities of this material.

5-Hydroxytryptamine did not interfere with either the BT or EE analyses since less than 0.5% of it was extracted into the organic solvent.

Examination of Table 2 confirms the direct influence of serotonin on quartered adrenals in other species. The adrenals from guinea pig, rat and cow responded to serotonin with a significant increase in BT material. However, it may be observed that this stimulatory influence was not as profound for the rat and cow adrenal as in the cases of the rabbit and guinea pig adrenals. The EE reducing material also increased and this was true for the cow adrenal too. Although the response of adrenal cortical slices from the cow is poorly discernible, it was felt that a shorter period between obtaining the glands at the slaughter house and performing the experiment would possibly improve the stimulation.

The influence of 5-hydroxytryptamine on adrenal homogenates was inspected and a species difference becomes obvious since rabbit adrenal homogenates responded to ACTH and serotonin while adrenals from guinea pig and rat did not. This would confirm the findings on ACTH and adrenal homogenates reported in a previous paper (3).

In an attempt to elucidate the nature of the BT material formed through the action of serotonin large scale incubations were performed. For both the pooled rat and rabbit adrenals which were not treated with serotonin traces of BT reactive zones could be detected on paper chromatograms. BT reactive zones were more readily detectable on paper chromatograms containing the residue from serotonin treated glands. For both the rat and rabbit the BT positive substances from the control incubations migrated at rates similar to the cortisol-aldosterone-cortisone type of compound and corticosterone, respectively. In a 16-hour chromatogram in the propylene glycol system, corticosterone had migrated approximately 35 to 40 cm. while the unresolved zone of cortisol, cortisone and presumably aldosterone occurred between 1–3 cm. The development of the chromatogram was held to 16 hours so that corticosterone would remain on the chromatogram. Extracts of the serotonin incubation showed no increase in the material migrating at the rate of corticosterone. The cortisol-aldosterone-cortisone zone contained the BT material which had increased in the presence of serotonin. Visual estimation indicated that 5 and 28% of the total BT material determined before chromatography was recovered for the rat and rabbit, respectively. These low recoveries in addition to the sensitivity of the original methylene chloride extracts to temperatures near 50° C suggested that the compound(s) was labile.

The toluene-chloroform-ethylene glycol system gave more definitive results. In this system cortisol migrated 5 to 8 cm. and cortisone 13 to 18 cm. after 16 hours. In both control and serotonin chromatograms, BT reactive zones were detected which corresponded to the mobility of cortisol, but only the serotonin chromatogram contained an intensely reactive BT zone between 9 to 12 cm. Chromatographic results were much less

clear for residues containing higher concentrations of lipids. In this situation the BT material that was increased by serotonin migrated over an extended area of the chromatogram with the lipids. Elution of the BT zone gave recovery values below 30%. The acetylation procedure of Mattox *et al.* (5), usually helpful in the isolation of aldosterone, did not yield any products.

It was hoped that larger pools of adrenal incubations would lead to isolation of the BT material. The residues from control and serotonin incubations of adrenal glands from approximately 100 rabbits were respectively chromatographed on silica gel columns. The results of a representative column chromatography are given in Table 3. It can be seen that the

TABLE 3. SILICA GEL CHROMATOGRAPHY OF CONTROL AND SEROTONIN RESIDUE FROM RABBIT ADRENAL EXTRACTS

Solvent	Control		Serotonin	
	Eluate wt.	BT*	Eluate wt.	BT*
	mg.	μg.	mg.	μg.
Benzene	59.9	340	16.6	40
Benzene	3.0	40	1.0	10
Benzene	1.1	10	0.6	6
Benzene-methylene				
Benzene chioride 20:1	0.9	12	1.1	4
Benzene chloride 5:1	0.9	10	0.5	14
Benzene chloride 1:1	0.3	11	0.9	10
Methylene chloride	0.5	16	0.6	6
Ethyl acetate	14.4	24	5.2	1100
Methanol	6.3	300	5.1	1050
Methanol	0.4	40	1.0	20
Total recovered from column	88.3	803	32.6	2260
Total added to column	90.2	922	32.6	2897

* The total μg. of BT material per eluate was estimated via a cortisol standard.

BT material elevated by serotonin occurred near the polar end. Approximately 80 to 90% of the BT material could be recovered and the BT material from control incubations concentrated in the methanol fractions whereas the serotonin released reducing substances partitioned between the ethyl acetate and methanol fractions.

Aliquots from the steroid fraction from the silica gel chromatography and corresponding zones from paper chromatography were submitted for electrolyte bioassay.[3] Eight 150 gm. adrenalectomized male rats received an intraperitoneal injection of 45 mg. NaCl and urines were collected for 6 hrs. Simultaneous bioassay was performed on a solvent control, 2 standards of deoxycorticosterone (DOC) and the unknowns. Slopes, test

[3] We express our deepest appreciation to Dr. Eugenia Rosemberg for carrying out the bioassays.

for parallelism and relative potencies were calculated according to accepted procedures. The results are given in Table 4. It was found that 0.037 and 0.094 μg. of the serotonin and control ethyl acetate fractions, respectively, were equal to 1 μg. of DOC. For the methanol fractions 0.051 μg. of the serotonin residue was equal to 1 μg. DOC while the control residue was inactive. These findings indicated that the serotonin ethyl acetate fraction was approximately 3 times as potent as the corresponding control fraction. Since the control methanol fraction did not exhibit any activity in sodium retention, the serotonin methanol fraction was strikingly more active.

TABLE 4. ELECTROLYTE BIOASSAY OF ETHYL ACETATE AND METHANOL FRACTIONS FROM SILICA GEL CHROMATOGRAPHY

Experimental groups	Dose*	Urinary sodium mean ± S.E.	Urinary potassium mean ± S.E.	Potasium/sodium mean ± S.E.
	μg.	mg./100 gm./hr.	mg./100 gm./hr.	
Control		2.44 ± 0.28	1.22 ± 0.64	0.52 ± 0.04
Deoxycorticosterone	6	1.30 ± 0.73	0.78 ± 0.05	0.60 ± 0.02
Deoxycorticosterone	25	0.77 ± 0.02	1.09 ± 0.04	1.41 ± 0.04
Control ethyl acetate	0.2	1.61 ± 0.11	1.06 ± 0.11	0.66 ± 0.01
Control ethyl acetate	1.0	1.09 ± 0.27	0.92 ± 0.12	0.90 ± 0.08
Serotonin ethyl acetate	0.2	1.27 ± 0.33	0.78 ± 0.14	0.71 ± 0.13
Serotonin ethyl acetate	1.0	0.80 ± 0.14	0.63 ± 0.06	0.72 ± 0.12
Control methanol	0.2	1.52 ± 0.33	0.77 ± 0.09	0.49 ± 0.07
Control methanol	1.0	1.85 ± 0.54	1.08 ± 0.14	0.67 ± 0.22
Serotonin methanol	0.2	1.45 ± 0.40	0.80 ± 0.16	0.61 ± 0.08
Serotonin methanol	1.0	0.87 ± 0.16	0.77 ± 0.10	0.91 ± 0.04

* Dose given in terms of BT reactive material.

DISCUSSION

These experiments have demonstrated a direct effect of 5-hydroxytryptamine on the adrenal cortex. The identity of the compound(s) released by the adrenal in the presence of serotonin escapes elucidation at the moment because of its lability. The nature of the non-steroidal reducing material measured by the ferric chloride-dipyridyl reaction also remains unknown. However, since metabolites of 5-hydroxytryptamine would probably retain a potential phenolic ring, they could give the EE reaction. Examination of 5-hydroxyindole acetic acid revealed that this major metabolite of serotonin does give a strong EE test but it is insoluble in methylene chloride and therefore could not account for the EE titers in the present study. Little or no stimulatory activity of 5-hydroxyindole acetic acid on rat adrenal was discerned.

As to the possible explanations for the activity of serotonin on the adrenal gland, two avenues of speculation are available. During hemorrhagic stress serotonin would be released from ruptured platelets. This sudden increment in serotonin titer might directly stimulate the adrenal to release aldosterone-type compounds as a method for altering electrolyte concentrations and combating deranged hemodynamics. It has already been observed that sodium retention occurred in humans receiving sero-

tonin (6) and chloride and water retention has been reported for the rat treated with serotonin (7).

A second possibility approaches an explanation revolving about the concentrations of precursors for corticosteroid synthesis and secretion. Bernheim *et al.* (8) have established that serotonin can function as an antioxidant and can inhibit the formation of lipid peroxides in rat brain homogenate and liver mitochondria. Other catechol and indole amines were much less active. The inhibitory activity of serotonin is particularly pronounced in the presence of ascorbic acid and it is well known that the adrenals are rich in vitamin C. Since cholesterol is a precursor of corticosteroids and can undergo peroxidation, then inhibition of this latter process would afford higher concentrations of precursors for corticosteroid synthesis.

If indeed it is aldosterone that is being released by 5-hydroxytryptamine then a possible common denominator for the varied effects of serotonin on smooth muscle, the kidney, blood pressure and brain activity is suggested. The intense sodium retention and potassium excretion caused by aldosterone could result in an abnormal electrolyte metabolism which might influence kidney function (e.g. reabsorption of chloride and water), osmotic pressure and heart action (e.g. blood pressure) and brain activity (e.g. depolarization of membranes). Since smooth muscle contains an intrinsic network of nerves, shifts in electrolyte concentrations can account for the increased contraction occurring in this tissue also because of polarization effects (7).

Acknowledgment

Our sincere appreciation is due to Miss Mary P. Kane and Mr. Roland Laferty for invaluable technical assistance.

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PROGESTATIONAL ACTIVITY OF 17-α-METHYL-19-NOR-TESTOSTERONE

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ABSTRACT

The progestational activity of methyloestrenolone and progesterone have been compared in immature female rabbits, using McPhail's method.

Small doses of either drug, when given intramuscularly result in endometrial hyperplasia of equal amount. However, larger doses of progesterone result in greater endometrial hypertrophy than similar doses of methyloestrenolone.

RECENTLY there has been much interest in the biologic effects of newer synthetic steroids including the progestational activity of methyloestrenolone (Orgasteron, Organon). The biological properties of this compound have been studied in women by Ferin (1) and in animals by Overbeek and de Visser (2). The former author has compared the progestational activity of methyloestrenolone to that of progesterone and ethynyltestosterone. Each of these compounds was administered sublingually. Luteoid activity was determined by the glycogen content of the endometrial cells. Ferin (1) found that methyloestrenolone was 150 and 50 times more active when administered sublingually than are progesterone and ethynyltestosterone respectively. Oral methyloestrenolone was found to be 10 times more effective than comparable doses of progesterone given intramuscularly.

The progestational activity of methyloestrenolone is apparently less in rabbits, as determined by the Clauberg test (3). Overbeek and de Visser (2) administered 1.0 mg. of methyloestrenolone *per os* in rabbits. Methyloestrenolone was almost as effective as 1.0 mg. of progesterone when given subcutaneously. These authors also studied the progestational activity in castrated pregnant rabbits and rats. They found that methyloestrenolone had a pronounced progestational property after oral administration. It was possible to maintain pregnancy in castrated animals, at least temporarily, but the fetuses sustained some damage. Toxicity was negligible.

In view of the results of the aforementioned investigators, the author has compared the activity of methyloestrenolone with progesterone when given intramuscularly.

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TABLE 1. EFFECT OF PROGESTERONE AND METHYLOESTRENOLONE ON ENDOMETRIUM OF IMMATURE RABBITS

Treatment	No. animals	Total dose (mg.)	Transverse endometrial surface area expressed in per cent of values encountered in control animals
No progestative drug	4	—	100
Progesterone	2	0.1	157
	2	0.5	201
	2	0.6	234
	4	2.0	254
Methyloestrenolone	2	0.1	155
	2	0.2	158
	2	0.3	160
	2	1.0	169
	4	2.0	180

MATERIAL AND METHODS

Methyloestrenolone was dissolved in propylene glycol (concentrations of 1:1,000 and 1:10,000). Progesterone was also prepared for intramuscular injection in a similar manner. Twenty-six immature female rabbits,[2] weighing 750–950 gm., were used. All 26 animals were given 25 I.U. of estradiol monobenzoate intramuscularly daily for 6 days. Four animals were used as controls. Ten animals received 0.1 to 2.0 mg. of progesterone. Twelve animals were given 0.1 to 2.0 mg. of methyloestrenolone. Each drug was administered intramuscularly daily for 5 days (see Table 1).

The four control rabbits were given 0.2 ml. of propylene glycol for the same period. On the day after the last injection of methyloestrenolone or progesterone, the rabbits were anesthetized with ether. The uterus of each was removed and fixed in Bouin's fluid for 24 hours. Sections were cut at 10 μ across the logitudinal axis of the uterus. The tissue was stained with hematoxylin and eosin. Progestational proliferation of the endometrium was determined by projection of the section on a screen. The total endometrial surface was measured with a planimeter (4, 5). The secretory epithelial area measurement represents a mean value based on many determinations of epithelial surface areas.

[2] A group of 16 rabbits were studied in the Laboratories of the Dept. of Obstetrics and Gynecology, University of Bologna, Italy, and 10 animals of a second group were studied at the Dept. of Obstetrics and Gynecology, University of Rochester, School of Medicine and Dentistry, Rochester, New York.

FIGS. 1–7. Cross section of uterus of immature rabbits. Hematoxylin-eosin. ×30.

FIG. 1. 150 I.U. of estradiol monobenzoate I.M. only (control).

FIG. 2. 150. I.U. of estradiol monobenzoate I.M. and 0.1 mg. of methyloestrenolone I.M.

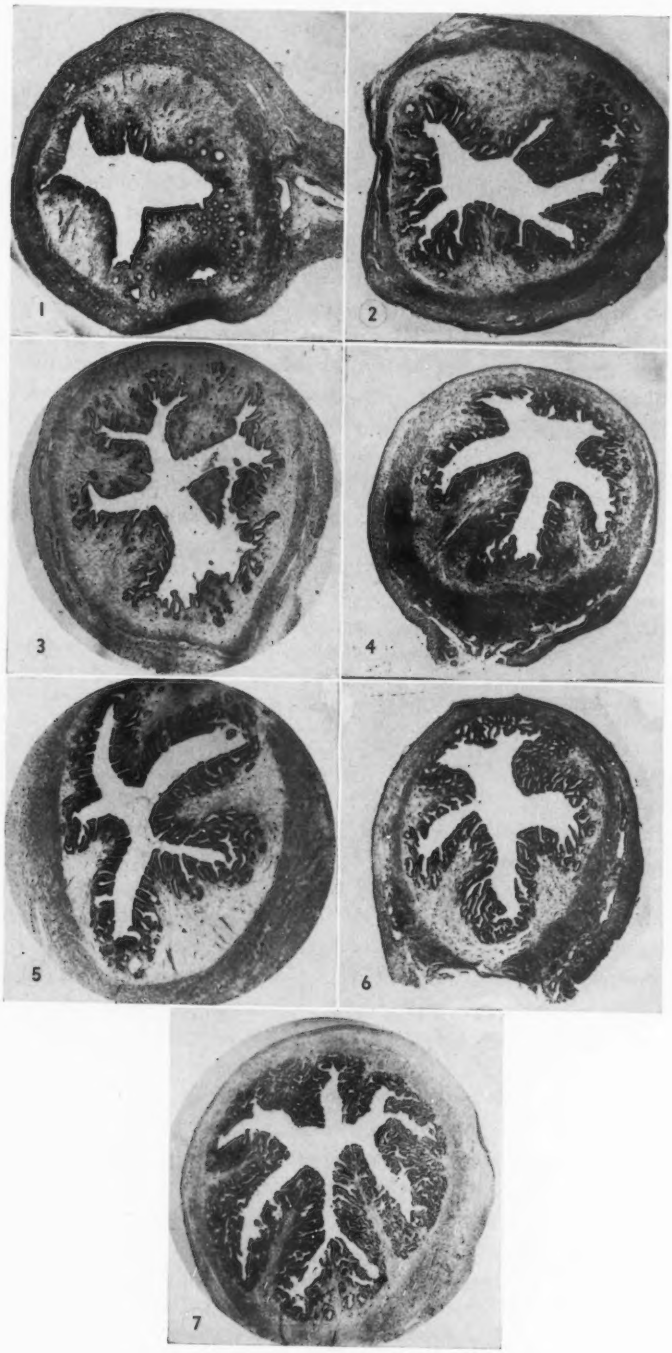
FIG. 3. 150 I.U. of estradiol monobenzoate I.M. and 0.1 mg. of progesterone I.M.

FIG. 4. 150 I.U. of estradiol monobenzoate I.M. and 0.2 mg. of methyloestrenolone I.M.

FIG. 5. 150 I.U. of estradiol monobenzoate I.M. and 0.3 mg. of progesterone I.M.

FIG. 6. 150 I.U. of estradiol monobenzoate I.M. and 2.0 mg. of methyloestrenolone I.M.

FIG. 7. 150 I.U. of estradiol monobenzoate I.M. and 2.0 mg. of progesterone I.M.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RESULTS AND DISCUSSION

A dose of 0.1 mg. of methyloestrenolone (given 0.02 mg. daily for 5 days) administered intramuscularly results in endometrial proliferation identical to that of similar doses of progesterone given intramuscularly. Larger doses of progesterone (0.5 to 2.0 mg.) result in much more endometrial hyperplasia than similar doses of methyloestrenolone (see Table 1).

The same results were found in all animals studied. The histological changes induced by each drug are illustrated in Figures 1–7.

The results of the author are thus unlike the findings of Ferin (1) and Overbeek and de Visser (2). The latter workers reported that methyloestrenolone had more progestational effect on the uterus than progesterone, when these drugs were administered orally. The author's results demonstrate that the progestational activity of methyloestrenolone and progesterone, when administered intramuscularly, are similar.

Therefore the comparative progestational activity of methyloestrenolone or progesterone is apparently dependent upon the route of administration as well as the test animal used.

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EFFECT OF THE INJECTION MEDIUM UPON THE BIOLOGICAL RESPONSE TO PARATHYROID HORMONE[1]

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ABSTRACT

Male Wistar rats weighing approximately 115–120 gm. were parathyroidectomized and maintained on a house diet. Four days later, after an initial blood sample was obtained, they received a known amount of parathormone A (purified hydrochloric acid gland extracts) or parathormone B (purified acetic acid extracts) either suspended or dissolved in one of several injection media. They were bled again three, six, nine, twelve, fifteen, eighteen, or twenty-four hours later. The concentration of calcium in the initial and final blood samples was determined, and the results plotted as increase in plasma calcium concentration versus time. It was found that 1) the duration of the biological response was dependent upon the injection medium, being longest when the hormone was given as a suspension in oil and shortest when given in isotonic saline; 2) the magnitude of the response, following the injection of PTH-B was dependent upon the injection medium, this effect being apparent as early as three hours after the test injection; and 3) N-ethyl maleimide in concentrations of 1×10^{-3} *M* or cysteine hydrochloride in concentrations of 1×10^{-1} increased both the magnitude and duration of the response to PTH-B given in isotonic saline. The implications of these findings are discussed.

IN A previous communication, we reported upon an alteration in the duration of action of parathyroid hormone after partial purification (1). This phenomenon was noted with partially purified hydrochloric acid extracts of bovine parathyroid glands having a potency of 75–100 U.S.P. units/mg. dry weight. As was expected, a similar phenomenon was observed upon purification of acetic acid extracts by the procedure previously described (2). Because this phenomenon is of obvious practical importance in the proper assessment of the potency of various parathormone preparations, it was decided to study it in more detail. The results of this study form the basis of the present report.

As has been pointed out (2, 3), the active principle in acetic acid extracts differs in certain regards from that in hydrochloric acid extracts of bovine parathyroid glands. In keeping with the terminology previously proposed (3), these principles will be called parathormone B (PTH-B) and para-

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thormone A (PTH-A), respectively, and these designations will be used throughout this paper.

MATERIAL AND METHODS

Male Wistar rats (CNF strain from Carworth Farms, N. Y.) weighing approximately 115–120 gm. were employed for all experiments. They were maintained on a normal house diet. Four days after parathyroidectomy, they were separated into groups of eight and used for the assays. Parathyroidectomy, anesthesia, heart puncture, and calcium determinations were performed as previously described (3). The animals were allowed free access to water but no food during the course of the experiments.

To test the effect of the various injection media upon the biological response, 0.25 mg. of PTH-B, prepared as previously described (2), or 0.50 mg. of PTH-A, prepared as previously described (3), was taken up in 0.5 ml. of the medium to be tested. After an initial blood sample was obtained by cardiac puncture, the hormone was administered subcutaneously in the right axillary region. A seocnd blood sample was collected from each rat of separated groups of 6 to 8 animals at intervals of 3, 6, 9, 12, 18, or occasionally 24, hours after the test injection. Usually 70 to 80 animals were employed in a given experiment. In some cases, the same group of six or eight rats was bled at 6 and then again at 18 hours, or 9 and 24 hours. Each set of experiments with a given injection medium was repeated at least once. At least one group in each series was given 0.25 mg. of PTH-B in 0.5 ml. of oil, and bled at 6 hours, to serve as a standard of comparison for successive series of experiments, such a procedure being the basis for the assay method previously described (3). The results from group to group were quite uniform after administration of the 0.25 mg. of PTH-B in oil. When given in arachis or sesame oil and assayed by the six-hour method previously described (3), the PTH-B preparations had an activity of 180–200 U.S.P. units/mg. dry weight, and the PTH-A 75–100 U.S.P. units/mg. dry weight. The results have been plotted as the increase in concentration of the plasma calcium versus time after injection. The results obtained with animals in which the initial calcium value was greater than 8.5 mg./100 ml. were not included because these animals were considered to have been only partially parathyroidectomized. The usual initial concentrations of calcium ranged from 6.0–8.5, and only three to four animals in each series of sixty to seventy had values greater than this.

The various solutions employed all were prepared upon the day of the assay, using "deionized" water and reagent grade compounds. The sesame or arachis oil was stored at 2° C and small aliquots were melted as needed. The oil was poured on to the dry hormone power and the mixture shaken vigorously to obtain a fine suspension. For the experiments with heparin the parathormone was taken up in saline or buffer and a drop of two of heparin added from a clean syringe. Upon the addition of the heparin a fine white cloudy precipitate formed and remained suspended in the solution. The gelatin employed was Type B "Pharmagel" gelatin of purest grade obtained from Knox Gelatin Co., Camden, N. J. An approximately 16% solution of this material was prepared by bringing 100 ml. of a pH 4.0 acetate buffer to a boil, removing it from the flame, and then adding 16 gm. of gelatin powder with constant stirring. After the solution had cooled to 40–50° C, 100 mg. of phenol were added and the material stored at 37° C, at which temperature the mixture was a highly viscous solution. None of the gelatin solutions were kept for more than one week, and were usually prepared the day before expected use. The N-ethyl maleimide and *l*-cysteine hydrochloride were obtained from Mann Research Laboratories, Inc., New York, N. Y. The human plasma, from a normal volunteer, was collected and stored with sterile precautions. The anticoagulant employed was heparin. Statistical analyses were carried out by standard methods (4).

RESULTS

In Figure 1 are shown the results obtained when the same preparation of PTH-A was taken up in saline, heparin solution or arachis oil, and injected into separate groups of animals. Each animal received 0.5 mg. of PTH-A in 0.5 ml. of the injection medium. Each point on the chart represents the mean response of 16 to 19 animals. Heparin or oil seem to be equally effective in prolonging the response. It is noteworthy that the response seen six hours after test injection is more or less the same regardless of the injection medium. This being the case, it is apparent that with a six hour assay procedure and these preparations an adequate assessment

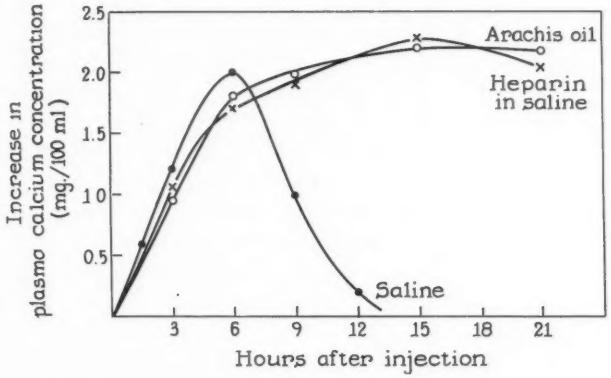

FIG. 1. The change in concentration of calcium in plasma of parathyroidectomized rats after the subcutaneous injection of 0.5 mg. of PTH-A in 0.5 ml. of heparin (×—×), oil (○—○) and isotonic saline (●—●).

of biological potency can be obtained using a variety of injection media. This is not the case, however, with the more highly purified PTH-B preparations as can be seen in Figures 2 and 3. In Figure 2 are plotted the responses observed when preparations of PTH-B (0.25 mg. per rat) were injected in oil or a pH 7.4 sodium bicarbonate buffer. Each point represents the mean response of 17–24 animals, and the vertical lines represent the standard errors. The most striking finding, other than the change in duration of action, is the observation that even as early as three hours after the injection there is a significantly greater response when the PTH-B is administered in oil rather than a neutral bicarbonate buffer. Furthermore, the results indicated in Figure 3 illustrate that a graded range of response is obtained depending upon the injection medium employed and the interval of time elapsed before measuring the response. Here again each point represents the mean response of 11–36 individual animals. The standard errors have been omitted for sake of clarity, but were similar

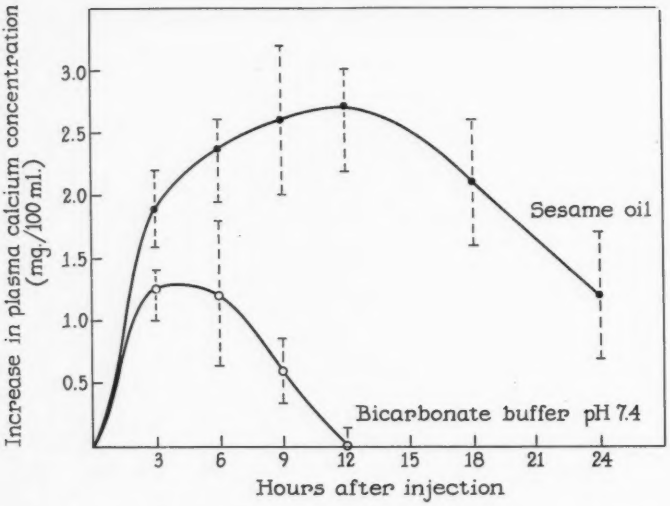

FIG. 2. The change in concentration of calcium in plasma of parathyroidectomized rats after the subcutaneous injection of 0.25 mg. of PTH-B in sesame oil (●—●), and a 0.1 *M* sodium bicarbonate buffer, pH 7.4 (○—○).

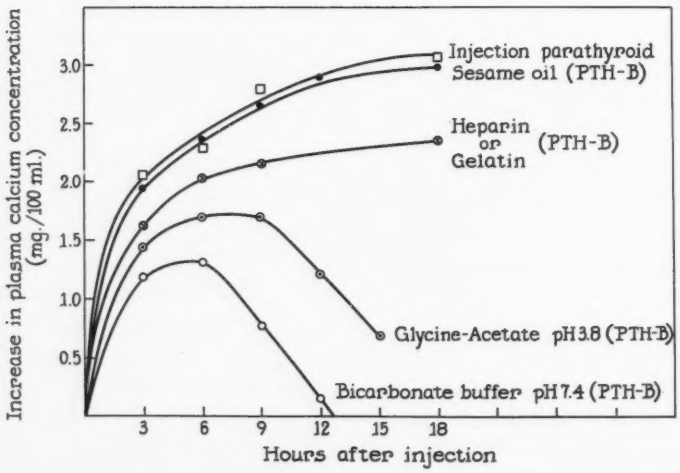

FIG. 3. The increase in concentration of calcium in plasma of parathyroidectomized rats following the subcutaneous injection of 0.25 mg of PTH-B in oil (●—●), heparin, gelatin solutions, or plasma (⊗—⊗), 0.01 *M* glycine acetate buffer, pH 3.8 (⊙—⊙), and 0.1 *M* sodium bicarbonate, pH 7.4 (○—○). The upper curve (□—□) represents the response obtained with 50 U.S.P. units of commercial Injection Parathyroid Hormone (Eli Lilly Company), or appropriate amounts of crude acid extracts of bovine glands.

to those shown in Figure 2. The response to 50 U. of Injection Parathyroid (Eli Lilly) has been included for comparison. A similar response is obtained when an appropriate amount of a crude acetic acid extract of parathyroid glands is injected in saline or oil. In these experiments (Fig. 3), in contradistinction to those with PTH-A (see Fig. 1), the response when the PTH-B was given in oil, rather than with heparin or gelatin, was significantly greater at nine or more hours after injection. The results at six hours, when analyzed statistically (4), were probably significantly different ($P > 0.01 < 0.05$). The response observed at 6 hours when the PTH-B is given in a 0.01 *M* glycine acetate buffer (pH 3.8) is significantly greater than when administered in a neutral bicarbonate buffer ($P < 0.01$).

A single set of experiments was carried out using sterile human plasma as the injection medium. The results obtained were similar to those obtained with the gelatin solutions and have not been plotted. It seemed possible that the heparin in the plasma was an important factor, so plasma obtained with a balanced oxalate solution was also tested during a routine six hour assay. The results were similar to those seen with the heparinized plasma.

Perhaps the most striking results are those shown in Figure 4. By adding 10^{-3} *M* N-ethyl maleimide or 10^{-1} *M* cysteine hydrochloride to saline both the magnitude and duration of the response to PTH-B is increased. The addition of 10^{-1} *M* cysteine hydrochloride to Injection Parathyroid (Eli Lilly) did not change the magnitude or duration of the biological response.

DISCUSSION

The significantly greater response obtained, even as early as three hours after injection, when PTH-B is given in oil rather than in a neutral bi-

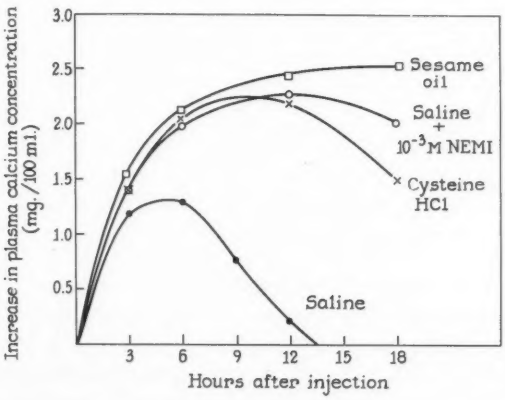

FIG. 4. The alteration in the response to the subcutaneous injection of 0.25 mg. of PTH-B produced by the addition of 10^{-3} *M* N-ethyl maleimide (○—○), or 10^{-1} *M* cysteine hydrochloride (×—×) to isotonic saline (●—●). The response obtained when this particular preparation was injected in sesame oil (□—□) is included for comparison.

carbonate buffer (Fig. 2) suggests that when given in the neutral buffer the PTH-B is partially inactivated at the site of injection. That this interpretation is more likely correct than the assumption that just a more rapid rate of absorption and excretion occurs is suggested by the results obtained with the addition of NEMI and cysteine hydrochloride (Fig. 4), as well as those obtained with glycine acetate buffer (Fig. 3). These results are in keeping with observations (unpublished) that PTH-B is unstable *in vitro* in neutral buffer, is stabilized by the addition of NEMI to these buffers, and is stable in glycine acetate and cysteine hydrochloride buffers.

Initially, it was thought that the effects of NEMI and cysteine hydrochloride could be explained by their known ability to prevent disulfide interchange (5). Further work (unpublished) has shown, however, that PTH-B has neither free SH groups nor contains cystine. Furthermore, it has been shown recently that parathyroid hormone loses biological activity when oxidized with hydrogen peroxide, and that subsequent reduction with cysteine hydrochloride results in the restoration of biological activity (6). In view of these results, it would appear that cysteine hydrochloride and NEMI exert their effect by inhibiting this oxidative inactivation of parathyroid hormone probably at the injection site. It seems possible that these two agents may also prevent the inactivation of the hormone at other sites since they both prolong the duration of action of the hormone. Studies are being undertaken to determine if such a synergistic effect exists.

The difference in the overall response to PTH-B when given in heparin or gelatin rather than oil (Fig. 3) may well be explained by a more rapid rate of absorption from the site of injection, and therefore a less sustained concentration of circulating hormone.

The results recorded in Figure 3 point out the difficulty encountered in assessing the biological potency of purified parathormone preparations; for, depending on the time period of assay used and the injection medium employed, one can find a potency of from 0–200 U.S.P. units/mg. of the same preparation. To date, the most reliable results have been obtained with oil as the vehicle for injection. When oil is employed the more purified preparations form a satisfactory, fine, even suspension, but the crude preparations do not disperse readily.

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IN VITRO AND *IN VIVO* METABOLISM OF PREDNISOLONE: STUDIES CONCERNING ITS BIOLOGICAL EFFECTIVENESS

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ABSTRACT

Metabolism of prednisolone by rat liver microsome-supernatant fractions has been reinvestigated. It has been found that both C-20 and Δ^4-3 ketone reduction of prednisolone is considerably slower than rates of reduction of these substituents on the hydrocortisone molecule. Comparisons of glycogenic activity also resulted in collection of data which suggest that prednisolone persists for a longer period in tissues or body fluids of fasted adrenalectomized rats. When equal amounts of either hydrocortisone or prednisolone were administered intraperitoneally to fasted adrenalectomized rats, it was found that forty-two times as much unaltered prednisoline appeared in the urine following a 48-hour collection period. The significance of these results in relation to increased biological efficacy of the synthetic steroid is discussed.

RECENT reports (1) suggest that rat liver slices do not metabolize prednisolone, 2-methylhydrocortisone, 9α-fluorohydrocortisone and 2-methyl-9α-fluorohydrocortisone at significantly slower rates than hydrocortisone. These results are in conflict with results reported from this laboratory (2) in which microsome-supernatant fractions of rat liver were employed. These results are also in conflict with studies reported by Schriefers (3) in which rat liver slices were used. In addition, Tompkins (4, 5) and Liddle (6) have reported that 2-methylhydrocortisone and prednisolone are metabolized more slowly than hydrocortisone by rat liver enzyme systems. Todd and Hechter (7) have demonstrated a slower rate of 9α-fluorohydrocortisone metabolism by liver paste and liver slices. *In vitro* studies, reported by Rongone (8), clearly indicate that in a bovine serum albumen preparation, in which a TPN-2H-generating system was supplied, the metabolism of both prednisolone and 9α-fluorohydrocortisone was significantly slower than that of hydrocortisone.

Slaunwhite, *et al.* (9) have shown that administration of prednisolone to human subjects results in excretion of more unaltered steroid in urine than that following administration of hydrocortisone. Vermeulen (10) and Gray (11) have clearly shown that considerable amounts of prednisolone appear in urine of patients treated with this hormone. These latter observations, coupled with those of Ely (12), which suggest that the biological half-life of prednisolone in human subjects is considerably longer than that of the

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parent hormone, tend to indicate that metabolism of prednisolone is significantly reduced.

Collins *et al.* (13), as well as Silber and Morgan (14) have shown that the biological half-life of prednisolone is longer than that of hydrocortisone following oral or intravenous administration to experimental animals (dogs). Glenn *et al.* (2) have shown not only an impeded rate of metabolism of 2-methyl-9α-fluorohydrocortisone by rat liver enzyme systems, but have also shown that intravenous administration of this compound to fasted adrenalectomized rats results in a markedly prolonged biological response —results which suggest that 2-methyl-9α-fluorohydrocortisone persists longer in tissues or body fluids. Critical analysis of the different types of data alluded to has fostered the concept (2) that slower rates of liver inactivation may partially explain the enhanced biological potency of certain synthetic steroids.

The following report is concerned with *in vitro* and *in vivo* studies concerning not only metabolism of one of the representative steroids under discussion (prednisolone), but also concerns itself with analysis of additional biological data which also tend to shed light on this subject.

METHODS

Methods for determination of steroid metabolism rates by isolated microsome-supernatant fractions of rat liver have been described in detail (2). Ability of steroids to induce liver glycogen deposition in fasted adrenalectomized rats was determined by previously described methods (15).

For urinary excretion studies, prednisolone and hydrocortisone were suspended in carboxymethylcellulose (5 mg./0.5 cc.) and injected intraperitoneally to fasted adrenalectomized rats. Animals were placed in metabolism cages; urine samples collected for 48 hours, pooled and subsequently extracted with five times volume of 20% ethyl acetate:chloroform. Solvent phases were extracted two times with 1/10 volume of 0.1N NaOH, 0.1N HCl, followed by distilled water extraction. Solvent phases were dried and, since dry residues appeared to be relatively free from extraneous material, total residues were dissolved in $CHCl_3$, transferred to paper strips, and chromatographed by the Bush (16) system. Steroids were identified by comparison of Rf values with standards, absorption spectrum in the ultraviolet, the Porter-Silber (17) method for detection of the dihydroxyacetone side chain, and H_2SO_4 chromogens in both ultraviolet and visible spectra. Determination of amounts of steroid present in eluates from paper strips was accomplished by ultraviolet and Porter-Silber methods of analysis.

RESULTS

When equimolar amounts (0.1 cc. of an 0.01 *M* solution) of hydrocortisone and prednisolone were incubated with isolated microsome-supernatant fractions of rat liver, it became readily apparent that both the Δ^4-3 ketone and C-20 ketone positions of the latter were reduced at considerably slower rates than the former (Fig. 1).

If a slower rate of liver inactivation of prednisolone is partially responsible for its increased glycogenic activity, one would expect a more prolonged

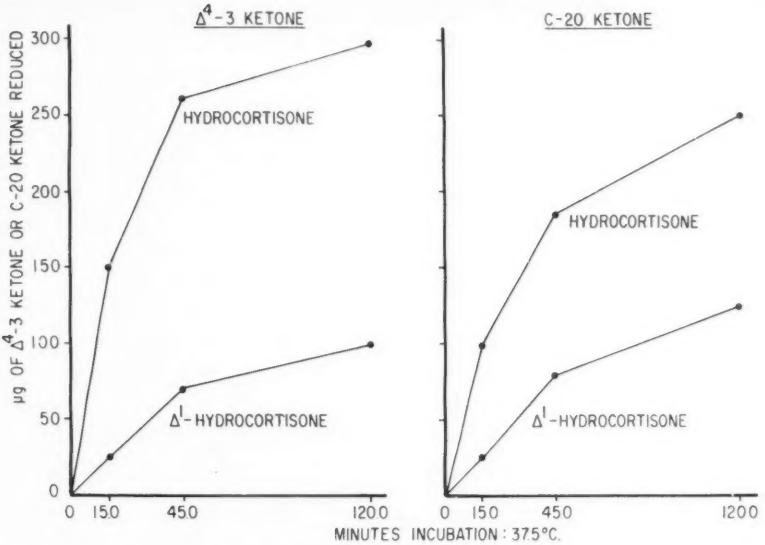

FIG. 1. Rate of Δ^4-3 ketone and C-20 ketone reduction of hydrocortisone and Δ^1-hydrocortisone (prednisolone) by isolated microsome-supernatant fractions of rat liver. (Each point: mean of duplicate determinations on duplicate samples.)

biological response following intravenous administration. When 1.0 mg. of prednisolone and 1.0 mg. of hydrocortisone (in 0.1 cc. EtOH) were administered intravenously (tail vein) to fasted adrenalectomized rats, results indicated that liver glycogen deposition response to prednisolone was more prolonged than response to hydrocortisone (Fig. 2).

Studies reported thus far indicate that prednisolone is metabolized more slowly by rat liver enzyme systems and, in keeping with this observation, a more prolonged biological response is obtained following its intravenous administration.

If metabolism of prednisolone is actually decreased in the fasted adrenalectomized rat, one would expect more of the "free," unmetabolized steroid to appear in urine of animals treated with this material. In order to be assured that both hydrocortisone and prednisolone would be equally exposed to liver inactivation, and in order to assure relatively complete absorption of both compounds, they were administered intraperitoneally. Hydrocortisone and prednisolone (10 mg./rat) were administered to two groups of twelve rats per group. Urine was collected over a 48-hour period, pooled, and analyzed according to methods described previously.

Following intraperitoneal administration of 120 mg. of hydrocortisone to twelve fasted adrenalectomized rats, 188 μg. of hydrocortisone were recovered in pooled urine samples. Following intraperitoneal administration

of 120 mg. of prednisolone, 7,860 μg. of free, unmetabolized substrate appeared in urine extracts. That is, forty-two times as much prednisolone appeared in the urine as did hydrocortisone. Since the amount of "free," nonmetabolizable steroid appearing in urine of animals treated with this hormone is probably a relative measure of reductive and conjugating mechanisms in liver, the conclusion seems warranted that liver of the fasted adrenalectomized rat metabolizes the steroid analogue less readily than it

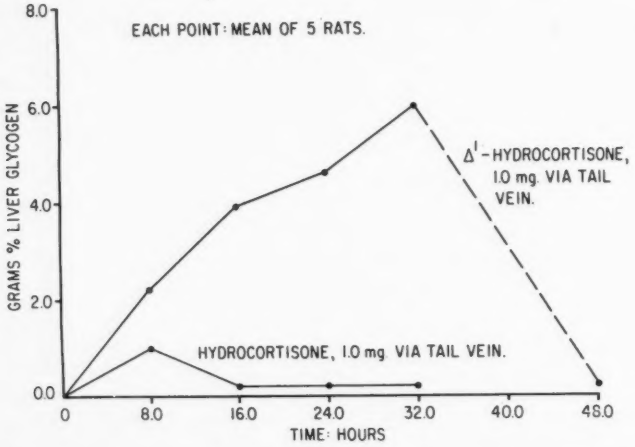

FIG. 2. Effect of intravenous administration of hydrocortisone and Δ^1-hydrocortisone (prednisolone) on liver glycogen deposition. Rats were adrenalectomized 3–4 days, fasted 24 hr. before injection, and the fasting continued to the end of the experiment.

does the parent hormone. Examination of peritoneal cavities of all rats at the conclusion of the 48-hour collection period revealed no evidence of incomplete absorption of either agent.

DISCUSSION AND CONCLUSIONS

Results of both *in vitro* and *in vivo* studies in the rat indicate that metabolism of prednisolone is considerably less efficient than that of hydrocortisone. *In vitro* results reported here are in essential agreement with those reported by Schriefers for rat liver slices (3).

Direct addition of steroid substr tes to liver slices and measurement of specific changes on the steroid molecule following one particular time period of observation is insufficient evidence for differences in metabolism of various compounds. Unless specific cofactor, coenzyme and cosubstrate requirements of a particular enzymatic reaction are thoroughly understood, differences, or lack of them, following addition of various materials mean very little with regard to the interpretation of significance of data. Certain subleties exist in experiments of this type, and acquaintanceship with vari-

ous analytical procedures involved are requisite for a thorough analysis of the multitude of factors involved in such a complex relationship.

Obviously, *in vitro* observations alone are insufficient evidence for the conclusion that a series of similarly related events occur in the intact organism. However, when such data are coupled with many other types of analyses, using the intact animal, a valid conclusion may be warranted.

The question of whether a decreased rate of steroid metabolism is solely responsible for enhanced biological activity of prednisolone remains unanswered. However, a slower rate of inactivation or excretion of compound from the organism necessarily implies an enhanced biological efficacy, since more steroid becomes available to responsive tissues for a longer period.

Difficulties may be encountered when attempts are made to analyze differences in either steroid metabolism rates or steroid biological effects when one rigidly applied artificial time period and steroid concentration is imposed upon the system being examined. For example, 2-methyl-9α-fluorohydrocortisone is thirty-three times more potent as a glycogenic agent than is hydrocortisone; prednisolone is three times more active. However, when all three compounds are administered *subcutaneously* in equimolar amounts (1 mg.) and livers analyzed for liver glycogen deposition 8 hours later, amounts of glycogen are approximately the same in all cases (18). One would be tempted to conclude that the three compounds are exactly equivalent on a biological basis. However, when analyzed as a function of *time*, prednisolone and 2α-methyl-9α-fluorohydrocortisone produce glycogenic responses which persist for many hours after glycogenic response to hydrocortisone has disappeared (18). Furthermore, when administered in "biologically equivalent" amounts (hydrocortisone, 1.0 mg.; prednisolone, 0.3 mg.; and 2α-methyl-9α-fluorohydrocortisone, 0.03 mg.), the glycogenic time curves become superimposable for these three steroids (18). These latter results further emphasize the probability that not only do retarded rates of steroid metabolism partially explain increased activity of certain steroids, but that other unknown mechanisms may be operating to bring about increased biological efficacy.

Acknowledgment

The author gratefully acknowledges the confirmatory analytical data collected by Mr. Lester Reineke concerning amounts of hydrocortisone and prednisolone excreted in the urine of rats following administration of each steroid.

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A METHOD OF ASSAY OF ANTITUMOR ACTIVITY USING A RAT MAMMARY FIBROADENOMA

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ABSTRACT

An assay method for investigation of factors which influence growth rate of a benign mammary fibroadenoma of female rats has been described. Variations in number of absolute tumor "takes," final tumor size and variable growth rates, necessitates employment of a definitely established, growing tumor for routine assay purposes.

Marked differences exist between response of mammary fibroadenomas to early treatment and treatment after tumors are growing autonomously. Since established tumors more nearly simulate the tumors dealt with in clinical medicine, studies of tumors of this type may provide correlations between laboratory and clinical observations.

Ovariectomy retards growth rate of established mammary fibroadenomas; adrenalectomy induces only a slight inhibitory effect; but both procedures combined result in greater inhibition of tumor growth.

Estradiol cyclopentylpropionate, at dose levels which inhibit growth rate of *initially* transplanted tumors, stimulates growth rate of *established* tumors.

Progesterone, which stimulates tumor growth if administered from day 1 of transplantation, has no effect on subsequent tumor growth if administration is initiated after tumors are well established.

Hydrocortisone, if given in amounts adequate to prevent body growth, has no effect on growth of established tumors. However, administration of similar amounts of the same hormone for 5 days prior to tumor implantation results in greater final tumor size 65 days later.

Testosterone propionate, reference standard for this assay, has been found to consistently inhibit tumor growth but never induces regression. Testosterone cyclopentylpropionate has a biphasic effect on tumor growth, stimulating growth at low doses, inhibiting at higher doses. Tumor inhibitory effects of testosterone propionate are accompanied by ovarian and adrenal involution and preputial gland and uterine stimulation.

Four groups of controls are required for an assay of this type: initial tumor implant weights (1 of every 10 implants), pretreatment controls (35 days following implantation), final controls (65 days following implantation) and hormonal controls (1.0 mg. of testosterone propionate each day from day 35 to day 65).

HUGGINS *et al.* (1, 2) recently described experimental conditions for study of a mammary tumor of female rats. This tumor, a benign mammary fibroadenoma, responds in rather consistent fashion to certain

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hormonal alterations and glandular extirpations. Since effects of hormonal therapy and glandular removal on mammary tumor development have been learned almost exclusively from studies in man, use of an experimental tumor which responds in similar fashion becomes an important adjunct to this area of research.

Huggins *et al.* (2) have reported that most steroid hormones capable of inducing retardation of mammary fibroadenoma development also possess estrogenic or androgenic properties. Studies were initiated in this laboratory to determine if response of benign mammary fibroadenomas to steroid hormones was merely a reflection of other characteristic hormonal responses or whether this type of tumor represented an analytical tool which would enable the investigator to discover steroidal antitumor agents which do not induce a characteristic pattern of hormonal activity in the host. That this latter possibility exists has been suggested by the work of Huggins *et al.* (3, 4) who showed the inhibitory influence of 3-methylcholanthrene on both the experimental tumor and on spontaneous neoplasms in man. 3-Methylcholanthrene administration does not induce the characteristic hormonal pattern of activity observed following administration of androgens or estrogens.

Benign mammary fibroadenomas occur spontaneously in 50–65% of all female Upjohn Wistar (5) and Sprague-Dawley (1) rats after the age of 18 months. Both types of tumor can be transplanted into females of the same strain. Transplants grow slowly, if at all, during the initial three weeks following transplantation (latent period) then begin to grow more rapidly in a progressive manner. Tumors do not grow readily in male rats, in hypophysectomized or ovariectomized females, except under extraordinary circumstances. Ovariectomy retards growth rate of the rapidly growing tumor; adrenalectomy has a slight inhibitory effect; and removal of both hormonal sources induces greater inhibition of tumor growth than either procedure alone. Growth rate of benign mammary fibroadenomas is also retarded by administration of estrogens or androgens in appropriate amounts.

METHODS

Since the percentage of animals in which transplanted tumors grow varies from one series of experimental animal to another, and since the number of "takes" per rat varies when multiple transplants are employed, procedures described by Huggins *et al.* (1) have been slightly modified in the interest of greater assay reproducibility.

The original tumors were obtained from Dr. Huggins' laboratory in February, 1957; were transplanted to a large number of female Sprague-Dawley rats and have subsequently produced a tumor strain used successfully in our laboratory for more than twelve months.

Female Sprague-Dawley rats (45–50 days old, 140–160 gm.) are anesthetized with 1.0 cc. of 1% cyclopal by intraperitoneal injection. Incisions are made in the back of each animal, and channels in the subcutaneous connective tissue made with surgical forceps to about ½ inch caudal to each front leg on both sides. One operator removes "seed" tumors (one "seed" tumor-bearing animal sacrificed for every one hundred recipients)

by cutting slices of uniform thickness and punching uniform pieces of tumor (70 ± 20 mg.) with nasal cutting forceps. Transplants (one on each side) are quickly inserted into previously made channels and incisions are closed with surgical clips.

The animals are housed in cages in constant temperature, constant humidity animal quarters. Animals are allowed tap water and Purina lab chow *ad libitum*. Two operators can carry out the entire operation described above on two to three hundred rats in $1\frac{1}{2}$ to 2 hours.

Tumors are allowed to grow for 35 days; animals are then palpated for actively growing tumors. Those with extremely small or no tumors are discarded; those with large tumors are set aside for subsequent "seed" tumors. The remaining animals are randomized in "haphazard" fashion by placing in large carrying cages, removing animals, and dividing into dosage groups of eight to ten animals. Test compounds are administered daily, orally or subcutaneously, as finely dispersed suspensions in 0.5 or 0.2 ml. of carboxymethylcellulose vehicle. Treatment is continued for 30 days, after which animals are sacrificed and autopsied. The eight to ten animals used for each experimental point usually provide 16–18 tumors of rather uniform size. Three groups of controls are used in every experiment: an *initial control*, ten tumor-bearing animals sacrificed at the end of the 35-day growth period; a *final control*, consisting of untreated animals in which tumors have grown for 65 days; and a *hormone control*, consisting of ten animals to which 1.0 mg./day of testosterone propionate has been administered subcutaneously for 30 days. The last control is to insure that the tumors in the experimental rats are hormone-sensitive and have not mutated to the Huggins "Class II" (3) hormone-insensitive fibroadenoma.

RESULTS AND DISCUSSION

A characteristic growth rate curve of the tumor is illustrated in Figure 1. It is apparent that growth proceeds slowly during the first 21 days following transplantation. The 35-day period was chosen for experimental studies because tumors are easily inspected visually and manually at this time.

That the benign mammary fibroadenoma has a variable growth rate, when data from one experiment are compared with those from another, is well illustrated in Table 1. These results were compiled from separate assays during a two-month period. It is evident that adequate numbers of control animals must be examined both at the end of the 35-day and 65-day growth periods for each experimental series.

In Table 2 the number of tumor "takes" for several series of animals during a three-month period is shown. It is evident that considerable variation (45–75%) in number of tumor "takes" exists. It is equally evident that with such variation in number of tumor successes, treatment of tumor-bearing animals from the beginning of the transplantation period becomes a difficult problem. That is, difficulties might arise in the interpretation of significance of data if, in an experimental group, the number of tumor "takes" were 75% and those of the control group only 45%. For this reason we have elected to determine the influence of various agents on growth rate of mammary fibroadenomas only when absolute assurance is made that all treated and untreated animals have one or two actively growing tumors. Occasionally we have had a complete failure of tumor "takes" in an entire series of experimental animals. Explanations for these complete failures are

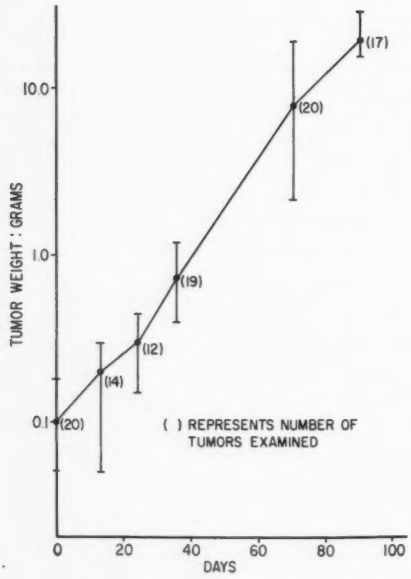

FIG. 1. Growth rate of mammary fibroadenoma.

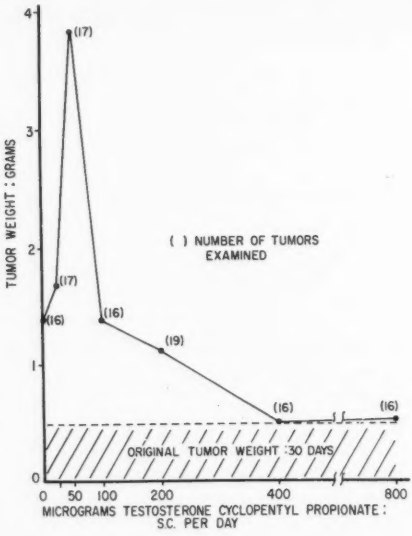

FIG. 2. Response of established mammary fibroadenoma to varying amounts of testosterone cyclopentylpropionate. Note: Final control tumor weights were lower than usual because these data were obtained in early days of investigations with tumor; at that time a rapidly growing "seed" tumor had not yet been discovered.

not available. However, their frequency does not obviate the use of this tumor as an analytical testing method.

Since the benign mammary fibroadenoma responds to androgenic agents, *testosterone propionate* was chosen as reference standard. In Table 3 data are shown to illustrate results obtained during a six-month period. It is obvious that testosterone propionate administration produces a consistent pattern of tumor inhibition (94–98%). Regression of the established mam-

TABLE 1. VARIATION IN SIZE OF MAMMARY FIBROADENOMAS 35 AND 65 DAYS FOLLOWING TRANSPLANTATION

Experiment No.	35-day tumors			65-day tumors		
	No. rats	No. tumors	Mean tumor weight (gm.)	No. rats	No. tumors	Mean tumor weight (gm.)
1040	10	19	.697	10	16	8.400
1037	10	19	.605	10	17	11.400
1035	10	16	.879	10	14	11.203
1034	10	17	.977	10	18	10.210
1032	10	17	.459	10	19	4.430
1028	10	19	.436	10	16	7.960

TABLE 2. VARIATION IN NUMBER OF TUMOR "TAKES" FOLLOWING TRANSPLANTATION OF BENIGN MAMMARY FIBROADENOMA

Experiment No.	No. animals	No. tumors transplanted	No. animals with tumors after 35 days	Per cent
1040	200	400	130	65
1037	200	400	90	45
1035	200	400	120	60
1034	200	400	102	51
1032	200	400	128	64
1029	200	400	135	67
1028	200	400	150	75
1026	200	400	95	47
1024	200	400	150	75
1020	156	312	107	68
1017	202	404	134	66
1015	200	400	155	77
1018	200	400	130	65
Total	2558	4116	1626	Avg. 64

TABLE 3. EFFECT OF TESTOSTERONE PROPIONATE ON GROWTH RATE OF MAMMARY FIBROADENOMA*

Treatment	No. animals with tumors (35 days)	No. takes 35 days	No. takes 65 days	Mean tumor weight (gm.)	Per cent inhibition
Initial Control	10	18		0.602	
Final Control	10		16	8.950	
Testosterone propionate†	10		17	1.030	94.9
Initial Control	10	19		0.356	
Final Control	10		17	4.420	
Testosterone propionate	10		20	0.568	94.8
Initial Control	10	19		0.913	
Final Control	10		17	6.070	
Testosterone propionate	10		18	1.040	97.9
Initial Control	10	16		0.950	
Final Control	10		15	10.210	
Testosterone propionate	10		14	1.130	98.9
Initial Control	10	15		1.062	
Final Control	10		14	17.880	
Testosterone propionate	10		11	1.250	98.9
Initial Control	10	17		0.977	
Final Control	10		13	10.200	
Testosterone propionate	10		20	1.180	97.9

* Selected at random from series of controls for period of 6 months.
† 1.0 mg./day for 30 days, subcutaneously.

mary fibroadenoma has never been observed following administration of testosterone propionate. Microscopic examination of tumors from testosterone propionate-treated animals reveals no significant alteration in morphological characteristics.[1] During a twelve-month period five animals

[1] Morphological examination of all tumors was made by Dr. John Clark, The Upjohn Company. Results will be published by Dr. Clark and the senior author in another communication.

from 202 testosterone-treated female rats were observed to possess resistant tumors.

A biphasic relationship exists between tumor development and dose of testosterone cyclopentylpropionate. That is, low doses of this particular steroid (25–50 μg./day) *stimulate;* higher doses *inhibit* tumor growth (Fig. 2).

Table 4 shows the effect of varying amounts of estradiol on growth rate of tumors and various end organs of female rats. It is evident that 0.2 to 48.0 μg./kg. of this material stimulates tumor development. The effect of estrogen administration under these conditions is different from that reported following estradiol cyclopentylpropionate administration beginning from day 1 of tumor transplantation (Fig. 3). These results may indicate differences in sensitivity of the established versus the newly-transplanted tumor. Larger doses of estradiol may be required to inhibit the established, rapidly growing tumor.

These latter results prompted a re-examination of possible differences between treatment of animals beginning at the time of tumor implantation and treatment initiated after tumors were established, i.e., growing autonomously. In Figure 3 results of treatment of tumor-bearing animals with various steroids are illustrated. All animals were treated from day 1 to day 90 following transplantation. It is important to note that 17-methyltestosterone, 9α-fluoro-11β-hydroxy-17-methyltestosterone (*Halotestin*) and estradiol cyclopentylpropionate (2 μg./day) all prevented this tumor from growing. The effect of estrogen under these conditions differs from that obtained when estradiol cyclopentylpropionate in equivalent amounts was administered following establishment of the benign mammary fibroadenoma (Fig. 4).

It will also be noted that amounts of hydrocortisone (2 mg./day, every

TABLE 4. EFFECT OF ESTRADIOL ON MAMMARY FIBROADENOMA DEVELOPMENT AND VARIOUS ENDOCRINE ORGANS OF THE FEMALE RAT

	No. tumors	BW gain (gm.)	Uterus (mg.)	Ovaries (mg.)	Preputial gland (mg.)	Adrenal (mg.)	Tumor wt. (gm.)	Per cent inhibition
Initial Control	19						0.559	
Final Controls	17	+16	443	109	77	58	7.460	
Estradiol								
0.05 μg./kg.	12	+24	467	110	108	68	7.980	+ 8
0.10 μg./kg.	12	+20	474	102	88	68	9.900	+36
0.20 μg./kg.	12	+19	478	118	69	64	15.570	+120
0.50 μg./kg.	12	+22	529	100	120	69	15.780	+121
1.50 μg./kg.	12	+26	440	93	86	62	15.040	+110
3.00 μg./kg.	15	+15	418	90	77	67	13.870	+93
6.00 μg./kg.	11	+ 7	441	86	83	64	14.120	+97
12.00 μg./kg.	14	+ 9	446	87	88	71	7.830	+ 5
24.00 μg./kg.	15	+13	623	73	99	74	10.020	+37
48.00 μg./kg.	13	0	759	63	81	67	9.090	+24
80.00 μg./kg.	14	− 4	688	56	97	68	6.340	−16

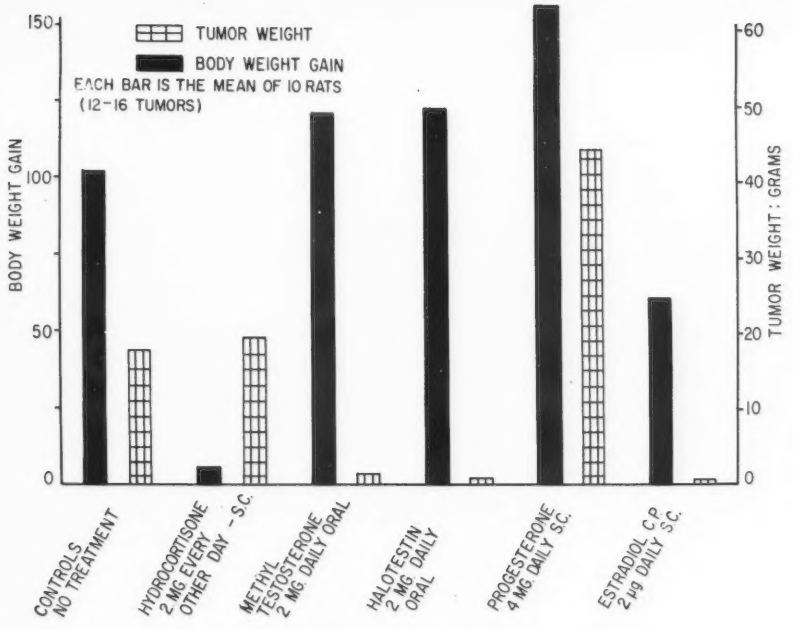

FIG. 3. Effect of various steroids on growth rate of the established mammary fibroadenoma.

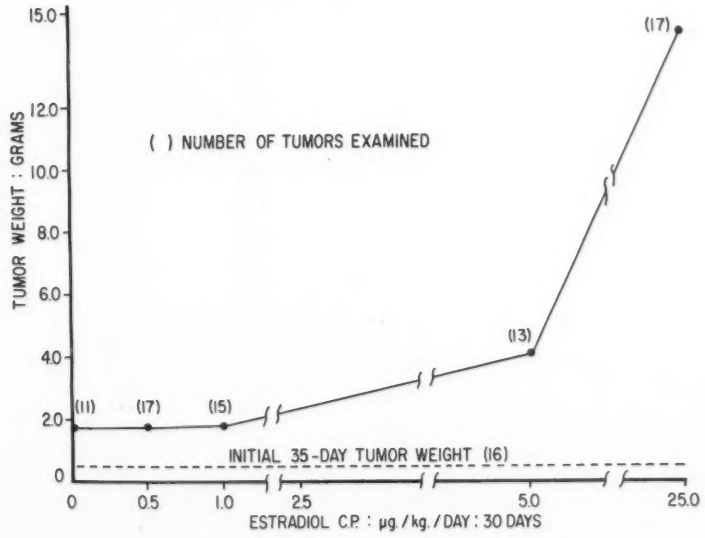

FIG. 4. Effect of estradiol cyclopentylpropionate on the growth rate of the established mammary fibroadenoma. Note: Final control tumor weights were lower than previous series because same slow-growing "seed" tumor from data in Fig. 2 used for initial implantation.

TABLE 5. EFFECT OF PRIOR ADMINISTRATION OF HYDROCORTISONE ON SUBSEQUENT TUMOR GROWTH

No. animals	No. tumors	Treatment	Tumor size	
			35 days	65 days
10	16	None	0.972	
10	18	None		20.200
20	38	2.0 mg. hydrocortisone per day, 5 days before implantation		51.530
10	17	2.0 mg. hydrocortisone per day, 5 days before implantation	0.875	

other day), sufficient to completely prevent body weight gain, had little influence on tumor growth. These results are in direct contrast to those obtained following administration of hydrocortisone *prior* to tumor transplantation. Hydrocortisone (2 mg./day) was administered for 5 days before tumor transplantation, then stopped; tumors were implanted on day 5 and examined 65 days later. Under these circumstances, tumors from hydrocortisone-treated animals were two to three times larger than control tumors from untreated animals (Table 5). These results are important from the standpoint of possible relationships which may exist between host resistance and subsequent mammary fibroadenoma development.

Results obtained with progesterone are in agreement with those reported

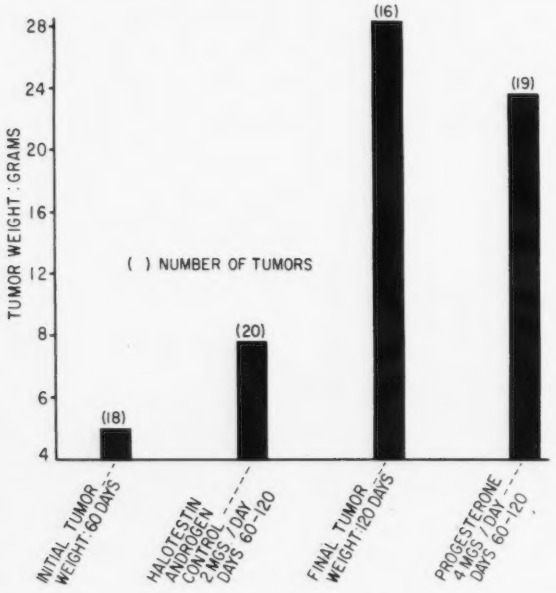

FIG. 5. Effect of progesterone administration on established mammary fibroadenoma.

by Huggins (1) who showed that treatment of animals with progesterone beginning at the time of tumor transplant resulted in considerable enhancement of tumor growth rate (Fig. 3). However, when equivalent amounts of progesterone were administered to animals bearing rapidly growing tumors (60 days following transplantation) for a period of 60 days (day 60–day 120), no effect was observed (Fig. 5). These results further emphasize differences which exist between treatment of animals with freshly-transplanted tumors and animals with well-established tumors. Either tumor growth is sufficiently autonomous or rapid during its later proliferative

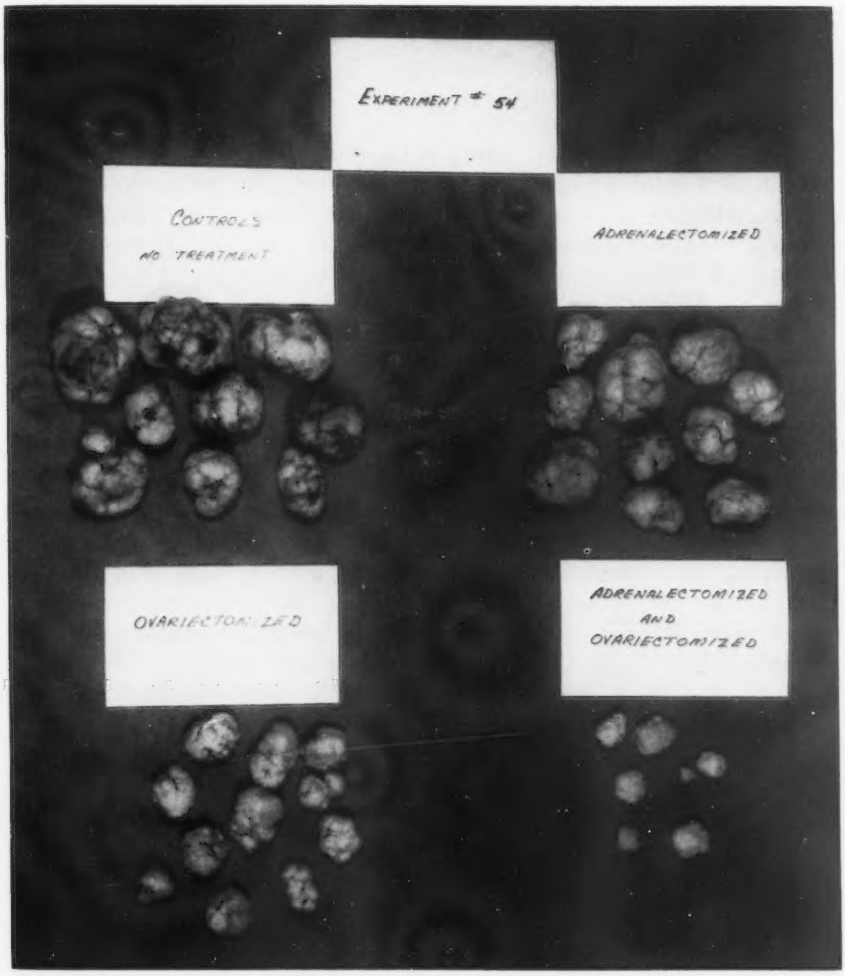

FIG. 6. Influence of adrenalectomy, ovariectomy and adrenalectomy plus ovariectomy on the growth of the established mammary fibroadenoma.

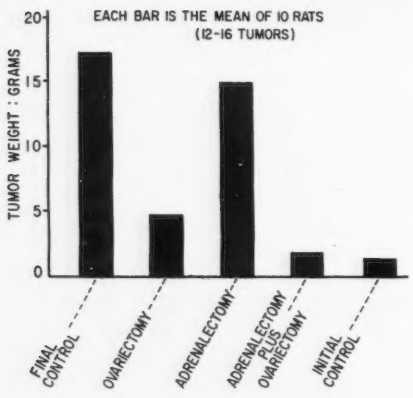

FIG. 7. Influence of adrenalectomy, ovariectomy and adrenalectomy plus ovariectomy on the growth of the established mammary fibroadenoma.

phases to prevent further influence by progesterone, or the pro-tumor effect of this agent is limited entirely to the host. This unknown effect may permit better establishment of tumor during its latent or early proliferative phases of development. However, prior administration of progesterone to experimental animals before tumor implantation (4 mg. for 7 days) did not result in greater number of tumor transplant "successes."

In Figures 6 and 7 results are presented to show the effect of removal of ovaries and/or adrenals on tumor growth. Adrenalectomized animals were maintained on 0.9% NaCl and 0.2 mg. hydrocortisone per day. It is evident that combined adrenalectomy-ovariectomy results in greater tumor inhibition. Neither procedure induces regression.

Testosterone propionate administration induces an increase in preputial gland growth while exerting an inhibitory influence on mammary tumor development (Table 6). The effect of varying amounts of testosterone propionate on ovaries, adrenals and uterus are also shown in Table 6. Simultaneous inhibition of both ovarian and adrenal secretory activity may explain, in part, the antitumor properties of this compound.

Since increased growth rate of preputial glands following administra-

TABLE 6. EFFECT OF TESTOSTERONE PROPIONATE ON TUMOR AND HORMONAL TARGETS OF FEMALE RATS

Testosterone propionate* (mg./kg./day, 30 days)	Tumor wt. (gm.)	Uterus wt. (mg.)	Preputial glands (mg.)	Adrenal (mg.)	Ovary (mg.)
0	8.210	582	99	62	90
0.5	5.420	280	152	62	49
1.0	4.890	339	161	51	38
2.5	0.850	820	229	48	44
5.0	0.620	901	321	48	56

* 10 rats per dose, 12–13 tumors per group of rats. Pretreatment tumor weight .621 gm. Testosterone propionate administered subcutaneously between days 35 and 65 after tumor transplantation.

TABLE 7. EFFECT OF TESTOSTERONE PROPIONATE ON GROWTH OF SEMINAL VESICLES AND LEVATOR ANI MUSCLES OF CASTRATE MALE RATS

Testosterone propionate (mg./kg./day, 20 days)	No. rats	Seminal vesicle (mg.)	Levator ani (mg.)
0.25	10	74	79
0.50	10	141	116
1.00	10	212	159
2.00	10	280	171
4.00	10	331	189
8.00	10	349	179
None	10	10	49

Note: These data were obtained following subcutaneous administration of testosterone propionate for 20 days to 27 day old castrate male rats (7).

tion of androgen is presumed to be an index of its "masculinizing" activity (6), the response of these organs appears to be the most critical or sensitive index of androgenicity. However, this statement is merely a working hypothesis and does not imply that for a compound to stimulate preputial gland development it must also be an androgen. Growth hormone and ACTH have also been shown to stimulate growth of preputial glands (8, 9). For comparative purposes, the androgenic and "myotrophic" activities of testosterone proprionate are illustrated in Table 7. Comparison of Table 6 with Table 7 reveals that amounts of testosterone propionate necessary to significantly inhibit mammary fibroadenoma growth rate are markedly androgenic and also significantly stimulate growth rate of preputial glands.

Acknowledgment

The authors are grateful to Dr. Charles Huggins for animals bearing the benign mammary fibroadenoma, for helpful advice during the initial phases of these experiments, and for reading this manuscript. We gratefully acknowledge assistance of Stanley C. Lyster in obtaining the data on androgenic activity of testosterone propionate.

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INHIBITION OF MAMMARY FIBROADENOMA OF FEMALE RATS BY STEROIDS OF THE ANDROSTANE SERIES

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ABSTRACT

Results have been presented to suggest that steroids can inhibit benign mammary fibroadenoma development without exerting significant androgenic or estrogenic effects in experimental animals. Preliminary observations suggest that the antitumor efficacy of these agents may depend upon their ability to inhibit certain secretory functions of the adenohypophysis. However, direct effects of steroids on tumor growth rate in the absence of the pituitary have not been determined. Final resolution of these questions awaits more detailed investigation in experimental animals and man.

Compared with 17-methyltestosterone, 11α-hydroxy-17-methyltestosterone and 9,11-epoxy-17-methyltestosterone have decreased androgenic activity while their antitumor activity is in a large measure the same. Compared with 19-nortestosterone, 2-methyl-19-nortestosterone has less so-called "masculinizing" ability in female rats, but antitumor activity is substantially the same.

Possible mechanisms regarding ability of these steroids to inhibit growth rate of mammary fibroadenomas in female rats cannot be stated with certainty at this time.

GROWTH rate of the Huggins tumor (a benign mammary fibroadenoma of rats) is retarded by a number of androgenic steroids (1, 2). This tumor will not grow in male rats, will not grow in hypohysectomized rats (except rarely), and growth rate is decreased by simultaneous removal of both adrenals and ovaries (3). The benign fibroadenoma has never been observed to undergo regression, nor are morphological characteristics significantly altered following administration of potent androgens (3). Growth rate is merely retarded by these agents. Growth rate is accelerated by administration of appropriate amounts of progestational and estrogenic steroids (2). However, these latter compounds do not markedly accelerate tumor growth when administered to hypophysectomized rats (2). If transplanted mammary fibroadenomas are allowed to grow for a number of weeks, followed by ovariectomy and/or hypophysectomy, they will stop growing but tumor regression is not induced by these procedures.

Huggins (2) has reported that most C-19 steroids which retard growth rate of the benign fibroadenoma also possess varying degrees of estrogenic

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or androgenic activity. Since hypophysectomy and ovariectomy retard tumor growth, an agent which effectively blocks ovarian or pituitary function, which also possesses minimal androgenic or estrogenic activity, would appear to be a desirable adjunct to treatment of patients with mammary carcinoma.

METHODS

A benign mammary fibroadenoma assay method has been described previously (3). Techniques used here are modifications of those recently reported by Dr. Huggins (1). Essentially, this procedure involves the use of an established tumor which has grown for 35 days prior to steroid administration. Two tumors were usually present in each experimental animal; 8–10 animals were employed for each experimental point. Control tumors were weighed 35 days and 65 days following implantation. These were designated initial controls and final controls respectively. An additional group, to which 1 mg. of testosterone propionate per day had been administered (day 35–day 65), was employed in every experimental series and was designated hormonal controls. Steroids were administered daily (day 35–day 65) either orally or subcutaneously as finely dispersed suspensions in 0.5 cc. or 0.2 cc. of carboxymethylcellulose.

Androgenic and "myotrophic" activities of steroids were determined by analysis of seminal vesicle and levator ani muscle weight changes in castrate male rats (4). Estrogenic activities of various steroids were determined by uterine weight changes in gonadectomized female rats.

RESULTS

Results obtained in the hormonal control group are indicated in Table 1. During the period in which the following experiments were undertaken (December, 1957–April, 1958) the benign mammary fibroadenoma was consistently responsive to testosterone propionate.

Testosterone propionate, at dose levels necessary to significantly retard tumor growth, also stimulates growth rate of preputial glands and uteri (Table 2). Growth of preputial glands of female rats following administration of androgens has been selected as an index of "masculinizing" properties of various compounds (5). That this assumption may not be strictly correct is indicated by studies which show that these end organs are also stimulated by growth hormone and ACTH (6, 7, 8, 9).

Investigations of antitumor activity of C-19 steroids revealed several interesting agents. Among these interesting compounds were 9,11-epoxy-17-methyltestosterone (U-5518) and 11α-hydroxy-17-methyltestosterone (U-5662). When compared with testosterone propionate, these two compounds inhibited mammary fibroadenoma development, but had no effect on uterine development (Fig. 1). 9,11-Epoxy-17-methyltestosterone did not stimulate growth rate of preputial glands at any dose level, while 11α-hydroxy-17-methyltestosterone did. Although the latter agent appeared to be a more potent antitumor agent than testosterone propionate, it did not stimulate growth of preputial glands as markedly as testosterone propionate (Fig. 1). Oral activity of these two compounds is illustrated in Figure 2. When compared to 17-methyltestosterone, U-5518 was equally

TABLE 1. EFFECT OF TESTOSTERONE PROPIONATE ADMINISTRATION (5 MG./KG.) ON GROWTH RATE OF ESTABLISHED BENIGN MAMMARY FIBROADENOMA

No. animals	No. tumors	Control tumors		Testosterone propionate (1 mg./day, 30 days)	Per cent tumor Inhibition
		35 Days (gm.)	65 Days (gm.)		
10	19	.478			
10	15		6.029		
8	15			.680	96.4
10	19	.592			
8	15		19.426		
8	13			.876	98.4
10	19	.557			
10	15		11.570		
8	16			.901	96.9
10	18	.816			
10	20		12.830		
10	20			.954	98.9
10	16	.980			
10	12		10.945		
10	18			.701	100.0
10	19	.557			
10	12		6.934		
10	15			.944	97.7
10	20	.473			
10	14		8.316		
10	14			1.794	83.2
10	15	.383			
10	15		9.160		
10	14			1.330	89.2
5	10	.458			
8	11		11.059		
8	12			1.203	92.9
10	19	.755			
10	20		3.082		
10	20			.761	100.0
10	19	.373			
10	14		2.744		
10	19			.568	91.8

TABLE 2. EFFECT OF VARYING AMOUNTS OF TESTOSTERONE PROPIONATE ON GROWTH RATE OF BENIGN MAMMARY FIBROADENOMA AND VARIOUS ORGANS OF INTACT FEMALE RATS

Dose mg./kg./day SC.	No. Animals	No. Tumors	Body weight gain (gm.)	Uterus (mg.)	Ovaries (mg.)	Preputial gland (mg.)	Adrenals (mg.)	Tumor wt. (gm.)	% Inhibition
Initial Controls	10	19						0.697	
Final Controls	8	13	+36	571	91	87	60	8.400	
0.5	8	16	+50	271	50	151	62	5.700	35
1.0	8	16	+54	384	36	158	53	5.360	40
2.5	8	12	+56	842	44	230	50	0.834	99
5.0	8	13	+55	900	52	307	48	0.720	100

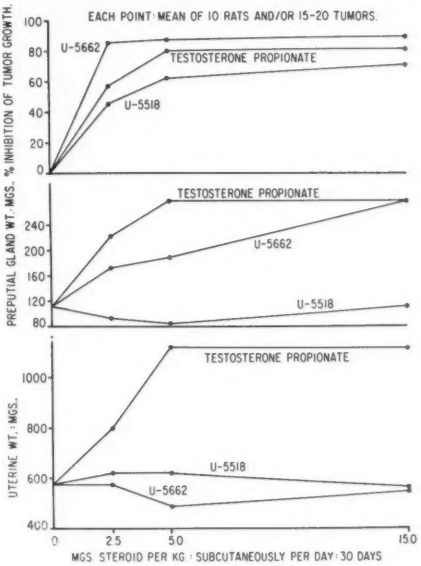

FIG. 1. Effect of subcutaneously administered testosterone propionate, U-5518 and U-5662 on growth rate of established mammary fibroadenoma, preputial glands and uteri of intact female rats.

as effective as an antitumor agent, but did not stimulate significantly growth rate of preputial glands.

Androgenic and "myotrophic" effects of these two steroids were compared in castrate male rats. Analysis of data shown in Figure 3 reveals that

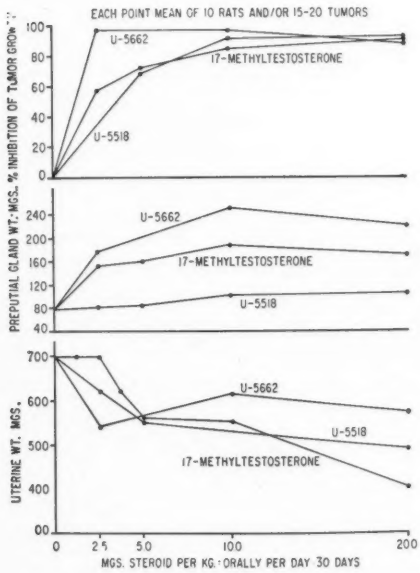

FIG. 2. Effect of orally administered 17-methyltestosterone, U-5662 and U-5518 on growth of established mammary fibroadenoma, preputial glands and uteri of intact female rats.

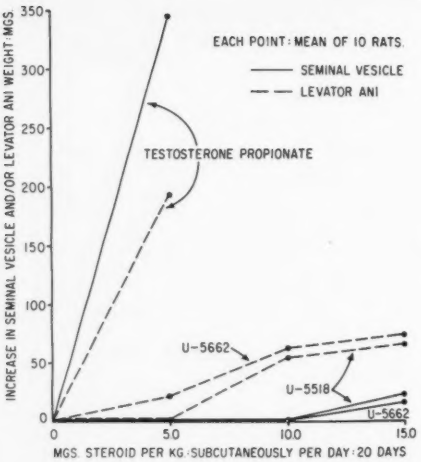

FIG. 3. "Myotrophic" and androgenic potency of testosterone propionate, U-5662 and U-5518.

both these steroids are less than 5% as active as testosterone propionate as androgenic or "myotrophic" agents. In addition, dose levels which produce significant anti-tumor activity in female rats were not androgenic in male rats (Figs. 1 and 3).

When administered at the higher dose levels (15 mg./kg.) to gonadectomized female rats, neither 9,11-epoxy-17-methyltestosterone nor 11α-hydroxy-17-methyltestosterone exhibited a significant degree of uterotrophic activity. However, the 11α-hydroxy compound augmented slightly the growth stimulatory effects of estradiol on the uterus (Table 3).

Since both 11α-hydroxy-17-methyltestosterone and 9,11-epoxy-17-methyltestosterone inhibited tumor growth without appearing to possess a significant degree of either androgenic or estrogenic activity, it was of interest to determine their effects on other end organs. Results of these experiments are illustrated in Table 4. It is apparent that both compounds significantly retarded growth of ovaries and adrenals of tumor-bearing female rats.

It has been demonstrated previously that 15 mg./kg. of either of these latter compounds is less than 5% as androgenic as testosterone propionate

TABLE 3. UTEROTROPHIC AND ANTI-UTEROTROPHIC ACTIVITY OF 11α-HYDROXY-17-METHYLTESTOSTERONE (U-5662) AND 9,11-EPOXY-17-METHYLTESTOSTERONE (U-5518)

Preparation	No. rats	Dose per day (20 days)	Uterus (mg.)
A CMC Vehicle	10	0.2 cc.	22
B Estradiol	10	0.8 μg./kg.	143
C U-5518	10	15.0 mg./kg.	23
D U-5662	10	15.0 mg./kg.	19
B+C	10		143
B+D	10		164

TABLE 4. EFFECT OF TESTOSTERONE PROPIONATE, 9,11-EPOXY-17-METHYLTESTOSTERONE AND 11α-HYDROXY-17-METHYLTESTOSTERONE ON GROWTH OF VARIOUS ORGANS OF FEMALE RATS

No. rats	Compound	Dose (mg./kg.) 30 days	Uterine wt. (mg.)	Ovarian wt. (mg.)	Preputial glands (mg.)	Adrenals (mg.)	Mean tumor wt. (gm.)
10	TP	5.0	779	95	306	60	.980
10	U-5662	15.0	389	82	208	51	.670
10	U-5518	15.0	440	78	102	48	.510
10	CMC Vehicle	—	520	116	92	72	9.700

Note: Initial tumor weight: .429 gm. (10 animals, 18 tumors).

at 5 mg./kg. (Fig. 3). However, when similar amounts were administered in these experiments, both of these compounds appeared to be slightly more active than testosterone propionate with regard to their ability to inhibit ovarian and adrenal function.

During the studies detailed above, several preliminary observations suggested that marked differences existed with regard to the effects of other steroids on growth rate of benign mammary fibroadenomas. At 15 mg./kg. 19-nortestosterone inhibited mammary fibroadenoma development, but also induced marked increases in preputial gland weight. However, at equivalent dose levels, the 2-methyl compound (2-methyl-19-nortestosterone) was as effective as an antitumor agent, but failed to stimulate significantly growth rate or preputial glands (Table 5). Further analysis of

TABLE 5. EFFECT OF 19-NORTESTOSTERONE, 2-METHYL-19-NORTESTOSTERONE AND TESTOSTERONE PROPIONATE ON GROWTH OF BENIGN MAMMARY FIBROADENOMA OF FEMALE RATS

Preparation	Dose (mg./kg.)	No. animals	No. tumors	Preputial glands (mg.)	Tumor wt. (gm.)	Per cent inhibition
Final Controls	—	8	11	99	8.375	—
19-Nortestosterone	15	8	15	317	.980	100
2-Methyl-19-nortestosterone	15	8	11	103	.820	100+
Testosterone propionate	5	8	11	279	1.130	98
Initial Controls	—	10	16	—	.950	—

these observations is illustrated in Figure 4. It is apparent that 2-methyl-19-nortestosterone is a potent antitumor agent with minimal activity with regard to its ability to stimulate preputial gland development. Its antitumor effectiveness may reside in its ability to inhibit pituitary gonadotropin secretion as reflected by decreased ovarian and uterine weight changes following its administration.

When 9,11-epoxy-17-methyltestosterone, 11α-hydroxy-17-methyltestosterone and 2-methyl-19-nortestosterone were administered at the 15 mg./kg./day level to weanling male rats, results indicated that 2-methyl-19-nortestosterone was an effective gonadotropin suppressor. 9,11-Epoxy-17-methyltestosterone and 11α-hydroxy-17-methyltestosterone did not demonstrate this degree of gonadotropin suppression (Table 5a).

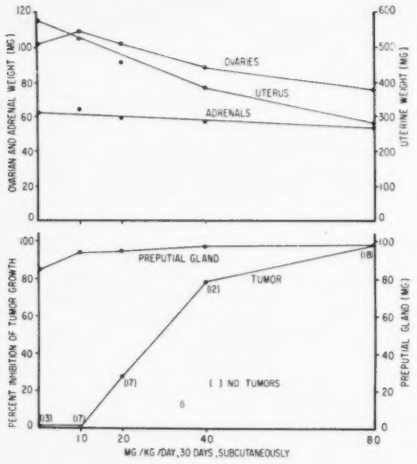

FIG. 4. Effect of 2-methyl-19-nortestosterone on growth rate of mammary fibroadenoma, preputial glands, uteri, ovaries and adrenals of intact female rats.

In order to further investigate the failure of 11α-hydroxy-17-methyltestosterone to inhibit gonadotropin secretion, it was administered in varying amounts to male weanlings for a 40-day period. When compared with testosterone propionate, it will be noted that 11α-hydroxy-17-methyltestosterone failed to inhibit testicular development (Table 5b). On the contrary, this steroid inhibited seminal vesicle growth rate, while testosterone propionate produced a marked increase in growth rate of this organ. These results are interesting with regard to antitumor effects of 11α-hydroxy-17-methyltestosterone which are significantly greater than those produced by testosterone propionate (Fig. 1). These latter results further emphasize the unique character of 11α-hydroxy-17-methyltestosterone.

Administration of 2-methyl-19-nortestosterone to female weanling rats resulted in marked depression of ovarian and uterine weights (Table 5c). These latter results also suggest that 2-methyl-19-nortestosterone is an effective gonadotropin suppressor at the particular dose levels employed.

Administration of 2-methyl-19-nortestosterone to castrate male rats

TABLE 5a. EFFECT OF 2-METHYL-19-NORTESTOSTERONE, 9,11-EPOXY-17-METHYLTESTOSTERONE, AND 11α-HYDROXY-17-METHYLTESTOSTERONE ON GROWTH RATE OF TESTES*

No. animals	Compound	Dose (mg./kg.)	Route	Testes (mg.)	Seminal vesicles (mg.)	Adrenals (mg.)	Body weight gain (gm.)
10	Controls	—	—	2274	269	33	113
10	9,11-Epoxy-17-methyltestosterone	15	SQ	2192	200	33	100
10	11α-Hydroxy-17-methyltestosterone	15	SQ	2187	201	30	117
10	2-Methyl-19-nortestosterone	15	SQ	1130	132	29	106

* Weanling male rats, 70–80 gm.; treated 40 days.

TABLE 5b. EFFECT OF TESTOSTERONE PROPIONATE AND 11α-HYDROXY-17-METHYLTESTOSTERONE ON GROWTH RATE OF SEMINAL VESICLES AND TESTES OF IMMATURE RATS*

No. animals	Compound	Amount	Route	BW gain (gm.)	Seminal vesicles (mg.)	Testes (mg.)
5	TP	50 μg./day	SC	220	641	2820
5	TP	100 μg./day	SC	237	663	2460
5	TP	400 μg./day	SC	218	1040	1170
5	TP	1000 μg./day	SC	188	1516	2250
5	11-OH-MT	50 μg./day	SC	236	489	2800
5	11-OH-MT	100 μg./day	SC	232	509	3010
5	11-OH-MT	400 μg./day	SC	218	344	2800
5	11-OH-MT	1000 μg./day	SC	237	418	2900
5	Vehicle	2 cc./day		226	629	3100

* Compounds administered for 40 days. Weanling male rats, 25 days old at beginning of experiment.

TABLE 5c. EFFECT OF 2-METHYL-19-NORTESTOSTERONE ON GROWTH RATE OF OVARIES, ADRENALS AND UTERI OF WEANLING FEMALE RATS*

No. rats	Compound	Dose	Route	Preputial (mg.)	Uterus (mg.)	Ovaries (mg.)	Adrenals (mg.)	Initial BW (gm.)	Final BW (gm.)
10	None	—	—	61	271	73	49	55	174
10	2-Methyl-19-nortestosterone	15 mg./kg.	SQ	57	139	27	34	53	214

* Compound administered in 0.2 cc. carboxymethylcellulose per day for 40 days.

(15 mg./kg.) indicated that this compound was less than 1% as effective as testosterone propionate as an androgen and less than 2% as effective as a "myotrophic" agent (Table 6). As a uterotrophic agent this compound was 0.001% as active as estradiol (Table 7). This degree of uterotrophic

TABLE 6. EFFECT OF 2-METHYL-19-NORTESTOSTERONE AND TESTOSTERONE PROPIONATE ON GROWTH RATE OF SEMINAL VESICLES AND LEVATOR ANI OF CASTRATE MALE RATS

Compound	No. rats	Dose—SC (mg./kg.)	Seminal vesicle (mg.)	Levator ani (mg.)
Vehicle	10	0.1 cc.	6	41
2-Methyl-19-nortestosterone	10	15.0	34	97
Testosterone propionate	10	0.25	74	80
	10	0.50	140	118
	10	1.00	210	150
	10	2.00	277	170
	10	4.00	320	185
	10	8.00	351	161

TABLE 7. EFFECT OF 2-METHYL-19-NORTESTOSTERONE AND ESTRADIOL ON UTERINE GROWTH OF GONADECTOMIZED FEMALE RATS

Preparation	No. rats	Dose (20 days, SC)	Uterus (mg.)
A Vehicle	10	—	22
B Estradiol	10	0.4 μg./kg.	106
C 2-Methyl-19-nortestosterone	10	15.0 mg./kg.	57
B+C	10		118

activity does not appear to explain entirely the effectiveness of 2-methyl-19-nortestosterone as an antitumor agent, since somewhat larger amounts of estradiol are required to produce *stimulation* of established tumor growth (Table 4, previous paper). Amounts necessary to produce tumor inhibition have not been ascertained for the established tumor, but, since 15 mg./kg. of 2-methyl-19-nortestosterone is less active than 0.15 μg./kg. of estradiol, its minimal activity as an estrogen does not appear to explain its antitumor effects.

That a steroid can have minimal "masculinizing" properties while exert-

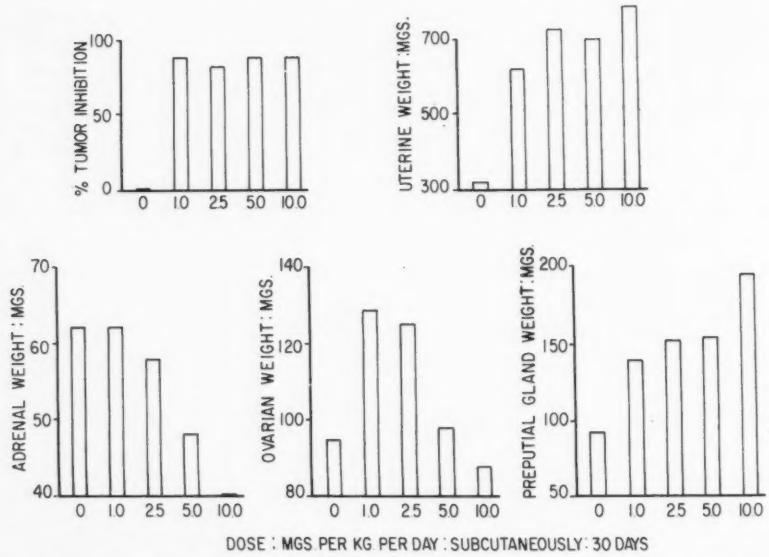

FIG. 5. Effect of 9α-fluoro-11β-hydroxy-17-methyltestosterone on uterus, ovaries, preputial glands, adrenals and benign fibroadenoma of intact female rats.

ing maximal effects on growth rate of mammary fibroadenoma of experimental animals is well demonstrated in Figure 5. These data resulted from administration of 9α-fluoro-11β-hydroxy-17-methyltestosterone (*Halotestin*)[1] subcutaneously to animals bearing the Huggins tumor. When compared to Figures 1 and 2 it is apparent that 9α-fluoro-11β-hydroxy-17-methyltestosterone exerts maximal anti-tumor effects at dose levels which induce less stimulation of preputial gland growth than 17-methyltestosterone or testosterone propionate. However, in contrast to 17-methyltestosterone, 9α-fluoro-11β-hydroxy-17-methyltestosterone causes a marked increase in growth rate of uteri of intact female rats.

[1] Registered trade mark, The Upjohn Company.

Acknowledgment

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THE MECHANISM OF INSULIN RESISTANCE IN THE KL STRAIN OF MICE: INACTIVATION OF INSULIN

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ABSTRACT

Insulin resistant (KL) mice, which tolerate 300–500 units of insulin without going into convulsions, inactivate insulin *in vivo* and *in vitro* at the same rate as control (BUB) mice which convulse after 50 units of insulin. The fasting blood sugar, glucose tolerance and pancreatic insulin content of the KL mouse is normal. After injection of small doses of insulin, the blood sugar response of the fed KL mouse is similar to that of the control BUB mouse. Following large doses of insulin, the blood sugar of control mice reaches the convulsive level within 2 hours. In the KL mouse, on the other hand, the blood sugar level declines but remains slightly above the convulsive level for 20–28 hours. These results are taken as evidence that insulin resistance in the KL mouse is not due to an increased insulinase activity, but to the ability of the animal to maintain a blood sugar slightly above the convulsive level for a long period after insulin administration.

MICE of the KL strain tolerate from 300–500 units of insulin without going into convulsions (1). Control BUB mice are more sensitive and convulse after doses below 50 units. The resistance to 300 units in the KL mouse is fully developed at 35 days of age, whereas older mice tolerate 500 units (1). Genetic studies suggest that the resistance may be due to about three pairs of additive recessive genes (2).

Previous investigations on the nature of insulin resistance in the KL mouse have shown that increased insulin excretion (3), increased glucagon secretion (4), or anti-insulin factors in the serum (5) cannot account for the insulin tolerance. Beyer (3) has found, however, that the liver of the KL mouse is capable of inactivating much larger doses of insulin than the liver of the control mouse. This increased *in vitro* insulinase activity of the liver is not shared by the kidney, spleen, muscle or blood of the KL animal (3).

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[2] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Syracuse University.

[3] Guidance and facilities for the isotope experiments in this investigation were provided by Dr. Harold F. Diermeier, Pharmacology Department, Upstate New York Medical Center, Syracuse, New York, for which the author is sincerely grateful.

A further investigation of this reported *in vitro* insulinase activity especially as related to *in vivo* activity, seemed advisable. If there were an increased *in vivo* insulinase activity in the KL mouse, one might expect a corresponding lack of circulating endogenous insulin and consequently a high blood sugar level, simulating a diabetic condition. Or, if the blood sugar of the KL mouse were normal, one might expect an increased rate of insulin secretion by the islets of Langerhans to counterbalance the high rate of insulin destruction. On the other hand, possibly the insulinase system is activated only after large doses of exogenous insulin. If such an "emergency response" is operating in the KL mouse, one would expect a normal blood sugar level and insulin concentration in the pancreas before insulin administration.

Our results indicate that, although a slightly increased rate of insulin destruction may exist, it cannot fully explain the insulin resistance of the KL mouse. The mechanism of insulin resistance more probably resides in the ability of the KL animal to maintain a blood sugar slightly above the convulsive level for a long period of time after large doses of insulin.

MATERIALS AND METHODS

All mice were bred and maintained in air-conditioned, properly humidified animal quarters. The insulin resistant (KL) animals[4] were screened at 40 days of age with an intraperitoneal injection of 200 units of insulin (Iletin, U-500).[5] During the screening procedure, food pellets were placed on the bottom of the cage. Control mice of the BUB strain[6] were used.

Destruction I^{131}-labeled insulin by rat liver extracts: The *in vitro* insulinase activity was assayed by the method of Mirsky *et al.* (6), but the liver homogenates were prepared in 4 volumes of 0.067 *M* phosphate buffer. One ml of the supernatant fluid was incubated with 1 ml. of an insulin solution, containing 0.027 unit of zinc-insulin (Iletin) and 0.5 microcurie of insulin-I^{131},[7] in a Dubnoff shaker at 38° C. As a control, the same amount of insulin was added to 1 ml. of phosphate buffer. After 30 minutes of incubation, the mixture was poured into tubes containing 2 ml. of 10% trichloroacetic acid (TCA), and centrifuged for 10 minutes at 18,000 ×g. The supernatant was diluted to 5 ml. with TCA and the precipitate dissolved in 5 ml. of 30% KOH. The radioactivity of 2 ml. aliquots from the supernatant and dissolved precipitate was measured with an end-window Geiger tube with a window thickness of 2.5 mg./cm.^{2}. Samples were counted in duplicate for 5 minutes and appropriate corrections for background counts were made. The per cent of total counts present in the supernatant fraction was used as a measure of insulin breakdown. The radioactivity in the supernatant of the control solution represented the small amount of non-precipitable insulin-I^{131} and was subtracted from the experimental values.

In vitro insulinase activity determined by the mouse bioassay technique: The liver homogenates were prepared as above in 3 volumes of cold 0.067 *M* phosphate buffer. After centrifugation the pH was adjusted if necessary to 7.5 with 1% KOH. One ml. of

[4] Originally from the colony of Dr. Herman B. Chase, Brown University.

[5] Generous quantities of U-500 zinc-insulin were supplied by the Lilly Research Laboratories, through the courtesy of Drs. O. K. Behrens and W. R. Kirtley.

[6] Originally from the colony of Dr. J. W. Wilson, Brown University.

[7] Prepared by Abbott's Laboratories.

the supernatant was incubated in a 37° C waterbath with 12 or 100 units of zinc-insulin (Iletin) for 30 minutes, and the mixture was shaken at 70 cycles per minute. As a control 1 ml. of insulin was incubated with 1 ml. of phosphate buffer.

For the insulin assay, an aliquot of the incubation mixture was diluted so that it contained a maximum of 0.05 unit per 0.25 ml. of solution. This was injected intraperitoneally into groups of 6 female Swiss albino mice[8] which had been fasted in individual metabolism cages for 16 hours. The bioassay animals were 4 months old and weighed 22–23 gm. The per cent of the initial blood sugar at 60 minutes was used as a measure of the amount of insulin left in the incubation mixture. Control mice received the diluted insulin-buffer solution (0.05 and 0.025 unit). The bioassay mice were re-used at intervals not shorter than one week. Groups were interchanged so that animals which received KL extract the first time were injected with BUB extract the next time.

The blood sugar was measured according to the Nelson-Somogyi method (7) on small samples (0.03 ml.) of tail blood. The volumes of all solutions were reduced proportionally.

Insulin-I^{131} destruction by intact mice: The *in vivo* rate of insulin destruction was measured by the technique of Mirsky *et al.* (8). Fed mice were injected intraperitoneally with 0.25 ml. of an insulin solution containing 10 units of zinc-insulin (Iletin) and 10 microcuries of insulin-I^{131}. They were sacrificed 60 minutes later and homogenized in a Waring Blendor for 8 minutes with 70 ml. of cold 10% TCA. All the glassware was kept cold before use and 1 mg. of bovine plasma albumin was added to decrease the adsorption of I^{131} to the glass (9). Part of the mixture was centrifuged and the radioactivity determined on 2 ml. aliquots of the whole homogenate and supernatant. Conditions and technique of counting were as described above. Samples were counted for 5 minutes or until at least 2,000 counts were recorded. The per cent of total counts present in the supernatant fraction in the form of I^{131}-labeled fragments was used as an index of insulin breakdown. To account for the amount of insulin destroyed during the homogenization process, one group of mice was killed immediately after the insulin injection and the degree of insulin breakdown in these control mice was determined.

Glucose tolerance: For the glucose tolerance tests, mice were fasted 16 hours in individual metabolism cages and injected intraperitoneally with 1 mg. of glucose per kg. bodyweight. The glucose was injected as a 10% saline solution and blood sugar values determined before and at intervals after glucose administration.

Pancreatic extraction: The pancreases of mice, fasted for 15 hours, were extracted according to the technique of Best *et al.* (10). The final precipitate, consisting of insulin and the hyperglycemic factor, was dissolved in isotonic saline (pH 2.5) of 30 times the volume of the original pancreatic weight. In most cases the pancreases of 2 animals were pooled.

Bioassay of the pancreatic extracts for its insulin content was carried out on fasted BUB male and Light female mice.[9] The age, sex and body weight of the bioassay mice in the experimental and control groups was matched. The average blood sugar drop of 6 mice during 60 minutes was used as a measure of insulin activity in the extracts. Extraction and bioassay of the experimental (KL) and control (BUB) pancreases were performed simultaneously. As a control for the bioassay, fasted BUB male mice were injected intraperitoneally with 0.4 ml. of acid saline and the blood sugar change determined. To estimate the amount of insulin extracted from the KL and BUB pancreas, standard doses of insulin, dissolved in isotonic acid saline, were injected into fasted BUB male mice and the drop in blood sugar determined.

Insulin tolerance: For the insulin tolerance experiments the mice were kept in individ-

[8] Purchased from the Albino Farms, Red Bank, New Jersey.

[9] Kindly supplied by Dr. H. B. Chase.

ual metabolism cages. Insulin was administered intraperitoneally and blood sugar levels were determined before and at intervals after insulin injection. All mice were allowed water and Purina Chow pellets *ad libitum* during the test.

RESULTS

Insulin inactivation: A preliminary determination of the *in vitro* insulinase activity with radioactive insulin was carried out in an attempt to confirm Beyer's findings of an increased insulinase activity in the KL liver (3). The results in Table 1, however, indicate that the per-cent of insulin-I^{131} breakdown by liver extracts of insulin resistant (KL) and control (BUB) mice is similar ($t = 0.7$; $P = .52$). Since a very small concentration of insulin was used in our incubation medium (0.027 unit) and since the increased insulinase activity may only manifest itself in the presence of

TABLE 1. PRELIMINARY DETERMINATION OF THE *in vitro* LIVER INSULINASE ACTIVITY OF INSULIN RESISTANT (KL) AND CONTROL (BUB) MICE

Strain	Sex	No.	Age (mo.)	Body wt. (gm.)	Liver wt. (gm.)	Av. counts/min. /aliquot		% insulin breakdown	Insulinase activity	Recovery (%)
						Precipitate	Supernatant			
KL	M	3	2–4	30	1.9	258	610	70	45 ± 0.9	94
BUB	M	3	2–4	29	1.7	277	617	69	44 ± 1.0	96
Control solution						674	223	25		

large doses of insulin, Beyer's experimental technique was repeated, using the bioassay method to measure *in vitro* insulin braekdown. Liver extracts were incubated with 12 or 100 units of insulin for 30 minutes and the amount of active insulin remaining in the incubation mixture was determined. Figure 1 represents the per cent of the initial fasting blood sugar of Swiss mice, one hour after the intraperitoneal injection of liver-insulin mixture. The average initial fasting blood sugar value of Swiss mice is 51 ±4 mg.%. The results in Figure 1 indicate that there is an equal amount of insulin left in the experimental (KL) and control (BUB) incubation mixtures, for the blood sugar drop in both groups of bioassay mice is similar. This implies that the *in vitro* inactivation of 12 or 100 units of insulin by the KL and BUB liver is similar. Blood sugar levels of bioassay mice injected with standard doses of 0.05 and 0.025 units of insulin (Fig. 1) indicate that approximately 50% of the insulin substrate is broken down during the 30 minute incubation period. We can conclude therefore, that the livers of insulin resistant (KL) and control (BUB) mice inactivate insulin *in vitro* to the same extent.

Since *in vitro* results may not accurately reflect *in vivo* conditions, the rate of insulin-I^{131} breakdown in intact animals has been determined. Intact insulin resistant (KL) mice do inactivate 10 units of radioactive insulin at

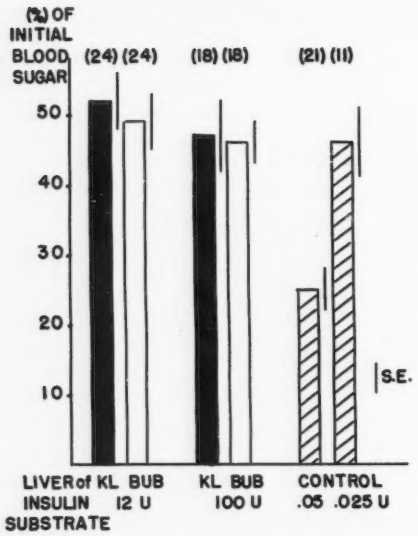

FIG. 1. *In vitro* insulinase activity of insulin resistant (KL) and control (BUB) liver, incubated with 12 and 100 units of insulin for 30 minutes at 38° C. ( ) indicates the number of bioassay mice.

a slightly higher rate than control (BUB) animals, but the difference is not significant at the one per cent level ($t = 2.3$; $P = .0344$) (Table 2). Increased rate of insulin destruction therefore cannot fully explain the insulin resistance in the KL mouse.

Glucose tolerance and pancreatic extraction: The fasting blood sugar levels and glucose tolerance tests of insulin resistant (KL) mice are similar to those of the control (BUB) strain (Fig. 2). If the degree of insulin destruction in the KL mouse were high, a corresponding increase in the rate of insulin secretion might be expected in order to account for the normal blood sugar levels. To test this possibility, pancreases of insulin resistant (KL) mice were extracted and the insulin content was determined by the bioassay technique. Figure 3 represents the blood sugar levels of BUB and Light bioassay mice before and one hour after the intraperitoneal injection of KL and BUB pancreatic extracts. The decreases in blood sugar

TABLE 2. INSULIN-I^{131} BREAKDOWN IN INTACT INSULIN RESISTANT (KL) AND CONTROL (BUB) MICE AFTER THE INTRAPERITONEAL INJECTION OF 10 UNITS OF INSULIN, CONTAINING 10 MICROCURIES OF INSULIN-I^{131}

Strain	No.	Sex	Age (mo.)	Body wt. (gm.)	Av. counts/min. /total sample		% insulin breakdown	Insulinase activity
					Homogenate	Supernatant		
KL	10	M	2–3	30	47,030	9,893	23.7	21.1 ± 1.6
BUB	9	M	2–3	30	42,923	7,999	18.6	17.1 ± 1.4
Control (KL+BUB)	3	M	2–3	30	48,376	777	1.6	

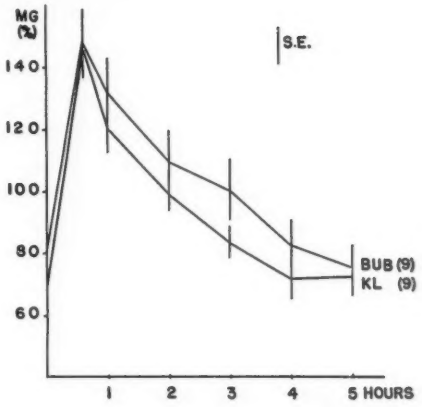

FIG. 2. Glucose tolerance of insulin resistant (KL) and control (BUB) mice. ( ) indicates the number of mice.

levels are the same and consequently the amount of insulin in the experimental (KL) and control (BUB) pancreatic extracts must be similar. By comparing the blood sugar drop after injection of pancreatic extracts with the blood sugar decline after known doses of insulin, it is apparent that 0.4 ml. of pancreatic extract contains approximately 0.01 unit of insulin. Since the dilution factor in this technique is 30, it appears that the pancreas of both the KL and BUB mouse contains approximately 0.75 unit of insulin per gram of tissue. This corresponds to the fasting values reported by Marks *et al.* (11).

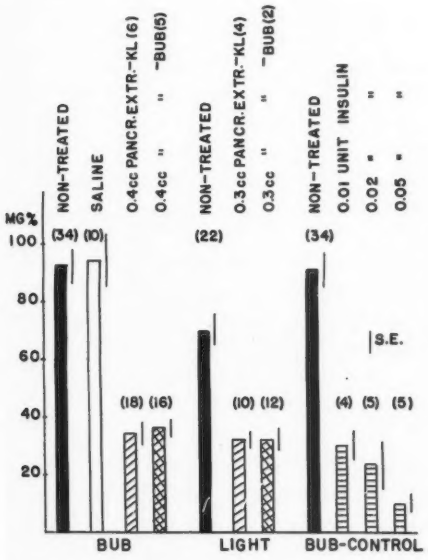

FIG. 3. Extractable insulin from the pancreas of insulin resistant (KL) and control (BUB) mice. Values represent blood sugar levels of fasted mice before and one hour after the intraperitoneal injection of the pancreatic extracts. ( ) indicates the number of mice.

Insulin tolerance curve: Insulin resistance could be due to a failure of insulin to exert its usual blood sugar lowering effect. That this is not the case in the KL mouse is indicated in Figure 4. The injection of one unit of insulin into fed KL mice is followed by a drop in blood sugar level, with a gradual return to normal in 4 hours. This blood sugar response is similar to that of control (BUB) mice (Fig. 4). After 200 units of insulin, however, the blood sugar in the KL mouse declines at about the same rate as after

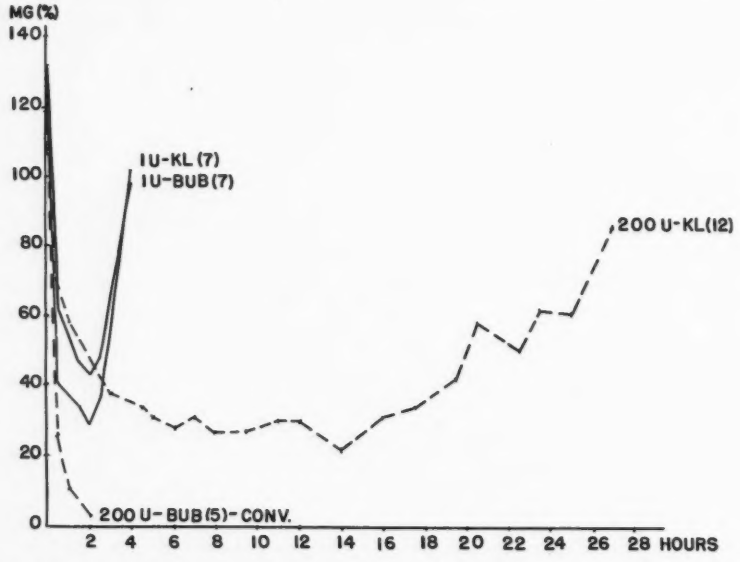

FIG. 4. Insulin tolerance of fed insulin resistant (KL) and control (BUB) mice after the intraperitoneal injection of 1 and 200 units of insulin. ( ) indicates the number of mice.

one unit, but remains at approximately a 30 mg.% level for 20 hours. Not until 27 hours after the insulin injection does the blood sugar level return to normal (Fig. 4). The blood sugar of the control BUB mice decreases rapidly and the animals convulse in 2 hours. These experiments indicate that insulin does have a blood sugar lowering effect in the KL mouse and that the insulin resistance mechanism probably resides in the ability of the KL animal to maintain a blood sugar slightly above the convulsive level for a long period of time.

DISCUSSION

The results reported here indicate that an increased rate of insulin destruction cannot fully explain the insulin resistance of the KL mouse, as is evident from the studies on insulin breakdown *in vitro* and *in vivo*. Using both the bioassay and the insulin-I^{131} technique, to assay insulinase

activity, it is clear that no difference exists in the rate of insulin breakdown between the experimental (KL) and control (BUB) groups of mice.

Indirect evidence for a normal rate of insulin degradation in the insulin resistant (KL) mouse is provided by the normal blood sugar values and pancreatic insulin content. If the rate of insulin breakdown were high, a correspondingly high blood sugar level or an increased rate of pancreatic insulin secretion would be expected to counterbalance the rapid rate of insulin destruction. As pointed out before, a normal blood sugar value, accompanied by a normal insulin concentration in the pancreas could also be interpreted in terms of an "emergency response." The insulin resistant (KL) mouse may increase its insulinase activity only after the administration of large doses of insulin. In that case, the untreated mouse would have a normal rate of insulin destruction and secretion, keeping the blood sugar balanced.

The emergency response, however, is unlikely in the light of observations of the blood sugar response of the KL mice after 200 units of insulin. Under these conditions, the blood sugar level declines to a value of 30 mg.% and remains at this approximate level for about 27 hours. If the rate of insulin breakdown in the KL mouse were high, or if the injection of 200 units of insulin would activate the *in vivo* insulinase system, the blood sugar level would be expected to return to normal values much earlier than is actually the case.

We feel that the mechanism of insulin resistance resides in the ability of the KL mouse to maintain a blood sugar slightly above the convulsive level for a long time after insulin administration, in contrast to a control mouse which allows the blood sugar to drop rapidly and reach the convulsive level. This result implies that the insulin resistant mice have access to a readily available carbohydrate source which they can utilize to counterbalance the insulin-induced hypoglycemia. This source does not necessarily have to be extensive since the animal maintains the blood sugar at only 20 mg.% above the convulsive level. Evidence for the contributing role of liver glycogen stores, food intake and intestinal absorption to the insulin resistance phenomenon in the KL mouse will be presented in a future report.

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AN EFFECT OF CORTICOTROPIN ON UREA FORMATION IN ADRENALECTOMIZED-NEPHRECTOMIZED RATS[1]

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ABSTRACT

Corticotropin has been found to share with growth hormone the capacity to diminish the accumulation of blood urea nitrogen consequent to the intravenous infusion of an amino acid solution (protein hydrolysate) into acutely adrenalectomized nephrectomized rats maintained with hydrocortisone. Milligram for milligram ACTH is as active as growth hormone. The nitrogen retaining action of ACTH is abolished by boiling in 0.1N NaOH but not by more gentle treatment with NaOH. ACTH activity is destroyed by oxidation with hydrogen peroxide but can be restored by incubation with cysteine, in keeping with other adrenal and extra-adrenal activities of ACTH. Growth hormone activity is not definitely destroyed by H_2O_2 treatment. It is concluded that the above action of ACTH on nitrogen metabolism is another extra-adrenal action of ACTH which is shared with growth hormone but cannot be accounted for by contamination of the ACTH with growth hormone, or vice versa.

RUSSELL and Capiello have reported that the administration to nephrectomized rats of both partially and highly purified preparations of growth hormone results in a significant decrease in the rate of urea formation following the intravenous infusion of protein hydrolysates (1, 2). In view of the recent claims from this laboratory and elsewhere that highly purified corticotropin exerts a number of metabolic actions in adrenalectomized animals which simulate those of growth hormone (3, 4, 5, 6, 7, 8, 9) an investigation of the influence of corticotropin on nitrogen metabolism was initiated using a modification of Russell's technique. At our instigation Russell has already tested a sample of oxycel ACTH and has reported that it does indeed have a growth hormone-like effect in the nephrectomized rat (10). The present study confirms and extends Russell's observation by demonstrating the effects of both growth hormone and ACTH in adrenalectomized-nephrectomized rats, thereby establishing the extra-adrenal nature of the response to the two hormones.

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MATERIALS AND METHODS

Twenty-four hour fasted male rats weighing from 200 to 250 grams were used. They were anesthetized with pentobarbital. Nephrectomy and adrenalectomy were carried out in one stage, following which the rats received 1 mg. of hydrocortisone hemisuccinate intraperitoneally. The rats were then placed in a chick incubator at 33° C to maintain body temperature and were kept there throughout the experiment except for blood sampling. Two hours postoperatively the test hormone was injected intraperitoneally, the controls receiving a corresponding volume of saline or 2 mg. of human serum albumin. Two hours later a tail blood sample was obtained, the animals were reanesthetized and 1 ml. of an amino acid solution (Parenamine) containing 15 mg. N was injected into the saphenous vein for each 100 gm. body weight. Two hours after this a second blood sample was obtained. The blood was analyzed for urea N by the method of Archibald *et al.* (11).

The characteristics of the hormones used and the methods of inactivation have been described in previous reports (5, 8). They included oxycel ACTH from the Wilson Company (lots 102201, 103322), α_1-α_2 ACTH from the American Cyanamid Co. and growth hormone from the Endocrinology Study Section of the National Institutes of Health (BGH-1).

RESULTS

Two sets of experiments were done, one in the summer of 1957 and the other in the spring of 1958. It will be noted in Table 1 that although qualitatively the results were comparable, quantitatively there was some variation between the response to the same hormone in 1957 and 1958. The table records the increase in urea nitrogen over the two hour period beginning with the amino acid infusion. In confirmation of Russell and Capiello growth hormone was found to lower significantly the urea accumulation rate compared to that of untreated controls. In the 1957 series the response was quite variable as indicated by the high standard error of the mean. The 1958 group was more consistent although the response to 1 mg./100 gm. was relatively small and was not increased when the dose was doubled.

Three samples of ACTH were tested. In 1957, 1 mg. of oxycel ACTH and 0.5 mg. of α_1-α_2 ACTH per 100 gm. body weight brought about depressions in urea formation which were of the same order of magnitude as has been found with growth hormone. These results were confirmed in the 1958 series with a different lot of oxycel ACTH. In this case the response was greater than to the corresponding dose of growth hormone.

We have previously reported that the extra-adrenal metabolic effects of ACTH are lost in parallel with its adrenal stimulating activity when the hormone is boiled in 0.1N NaOH or oxidized with H_2O_2 (5, 6, 7, 8). In the latter case activity may be restored in part at least by treatment of the oxidized hormone with cysteine. Growth hormone activity is destroyed by mild treatment with alkali (0.1N NaOH for 24 hours at 26° C) (10) but ACTH resists this treatment (5). The results of comparable studies measuring urea accumulation are found in Table 1. Oxycel ACTH boiled in 0.1N alkali lost activity; when subjected to the more gentle alkali treat-

TABLE 1. INFLUENCE OF GROWTH HORMONE AND ACTH ON RISE IN BLOOD UREA N DURING 2 HOURS FOLLOWING INTRAVENOUS INFUSION OF 15 MG. OF AMINO ACID N/100 GMS. BODY WEIGHT IN ADRENALECTOMIZED-NEPHRECTOMIZED RATS

Experimental groups	No. of rats	Rise in urea N in 2 hrs. mg.% (Mean ± S.E.)	P vs. control
Control—1957	21	20.42 ± 1.05	—
G.H. 1 mg./100 gm.	6	11.82 ± 2.25	< .01
G.H.-H_2O_2 treated	7	14.89 ± 1.12	< .01
Oxycel ACTH 1 mg./100 gm.	13	13.09 ± 1.02	< .01
Oxycel ACTH 0.1N NaOH, 26° C, 24′	7	15.80 ± 0.88	< .01
Oxycel ACTH 0.1N NaOH, 100° C, 20″	7	18.02 ± 2.25	N.S.
α_1-α_2 ACTH, 0.5 mg./100 gm.	6	13.49 ± 1.16	< .01
α_1-α_2 ACTH, 0.75 mg./100 gm., H_2O_2 treated	6	17.61 ± 1.31	N.S.
α_1-α_2 ACTH, 0.75 mg./100 gm., H_2O_2-cysteine	6	16.59 ± 0.92	N.S.
Control—1958	34	20.19 ± 0.55	—
G.H. 1 mg./100 gm.	9	17.14 ± 0.54	< .01
G.H. 2 mg./100 gm.	7	17.10 ± 0.76	< .01
G.H. 2 mg./100 gm., H_2O_2 treated	11	18.64 ± 0.99	N.S.
Oxycel ACTH—1 mg./100 gm.	15	15.69 ± 0.48	< .01
Oxycel ACTH—1 mg./100 gm., 0.1N NaOH, 100°, 20″	6	18.35 ± 1.10	N.S.
Oxycel ACTH—H_2O_2 treated	13	19.58 ± 0.85	N.S.
Oxycel ACTH—H_2O_2-cysteine	13	17.65 ± 0.72	< .02
Oxycel ACTH—2 mg./100 gm.	7	15.03 ± 0.68	< .01
Oxycel ACTH—H_2O_2 treated	9	20.77 ± 0.99	N.S.
Oxycel ACTH—H_2O_2-cysteine	8	14.28 ± 0.85	< .01

ment it retained its effect. Hydrogen peroxide treated ACTH was invariably inactive. Reactivation of the α_1-α_2 ACTH (0.75 mg./100 gm.) was not accomplished in 1957. On the other hand in the 1958 series some reactivation of oxycel ACTH was achieved at the 1 mg./100 gm. dose and unequivocal reactivation at the 2 mg./100 gm. dose. In the former case the urea rise of 17.65 ± 0.72 mg.% in the cysteine treated group was significantly less than the control ($P < .02$), but still not significantly less than the H_2O_2 treated hormone (19.58 ± 0.48 mg.%). We have previously found in other studies that cysteine reactivation may not be complete and hence that it is necessary to use maximal rather than minimal doses to demonstrate the phenomenon (5, 7, 8). In contrast to the results with ACTH, treatment of growth hormone with H_2O_2 did not lead to clearcut inactivation. In the 1957 series the H_2O_2 treated growth hormone was clearly active. In the 1958 series the results were indecisive since the urea rise from the H_2O_2 treated hormone was not significantly different from either the untreated hormone or the control. However, it should be noted that in contrast to the ACTH effects, the response to growth hormone was small to begin with and hence it is difficult to compare effects of various treatments of the hormone.

DISCUSSION

These results confirm the preliminary report of Russell of a nitrogen retaining effect of ACTH in nephrectomized rats which is comparable to that reported by her for highly purified growth hormone (2). In the pres-

ent report the extra-adrenal nature of the response is established by the use of hydrocortisone maintained acutely adrenalectomized rats. Milligram for milligram ACTH is at least as active if not more active than growth hormone, although as emphasized in previous papers, the doses of ACTH used are huge when expressed in units. The response of ACTH to various inactivation and reactivation procedures corresponds with what we have reported for other adrenal and extra-adrenal actions of ACTH (5, 6, 7, 8). In view of the dose relationships and the fact that growth hormone was not clearly inactivated by H_2O_2 treatment whereas ACTH was, one can definitely rule out the possibility that the response to the ACTH samples used was due to contamination with growth hormone. It might be noted in passing, too, that we have found H_2O_2 treated growth hormone to be fully active when tested by its ability to induce a positive nitrogen balance in tube-fed rats (13). Conversely, since growth hormone is inactivated by mild alkali treatment (10, 12) whereas ACTH is not, the very unlikely possibility that the growth hormone effects were due to ACTH contamination need not be seriously considered. The same reasoning also makes mutual contamination of both hormones by a common third factor rather unlikely.

These results can be taken as additional evidence that ACTH shares certain activities with growth hormone at least when the former hormone is administered in very large doses. The physiological significance, if any, of the activity described in this report is as yet unclear. Attempts to induce positive nitrogen balance in tube-fed adrenalectomized rats by the administration of ACTH have so far been unsuccessful, although growth hormone, as expected, is quite active. Further investigation of possible growth promoting effects of ACTH in the absence of the adrenal gland is clearly indicated.

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EFFECT OF FREQUENT HOUSING CHANGES AND OF MUSCULAR EXERCISE ON THE THYROID GLAND OF MICE[1]

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ABSTRACT

The possibility that the psychological stimulation resulting from frequent changes of housing might have an effect on the thyroid was investigated in mice "transferred" daily to different types of cages for a period of 8 weeks. Similarly, the effect of forced muscular exercise was investigated in mice made to walk daily 3½ to 4 hours in a rotating cage for a period of 8 weeks. All animals were fed controlled diets with low or adequate levels of dietary iodine.

Control mice on the low iodine diet had enlarged thyroids showing histological signs of stimulation. "Transferred" or exercised mice fed the same diet failed to show thyroid stimulation. Thus, frequent housing changes and forced muscular exercise prevented the thyroid stimulation resulting from iodine deficiency. This depressing effect on the thyroid does not appear to be due to unspecific stress, since adrenal weight was not altered.

IT IS widely held that human hyperthyroidism may arise from acute or chronic psychological stimulation (1, 2). However, acute psychological stimulation was shown by Brown-Grant *et al.* (3, 4) to produce an immediate decrease in the rate of release of radioiodine from the thyroids of immobilized rabbits. Such a decrease may be the result of associated stress since it is known that stress depresses the activity of the thyroid gland (5). Therefore, an experiment was designed to investigate the effect of psychological stimulation on the thyroid, using a stimulus mild enough to avoid stress. Another stimulus, namely forced muscular exercise, has also been reported to depress thyroid activity (5). However, this effect was slight as compared with that of other stressing agents which produced an intense depression of the thyroid gland. It was possible that forced muscular exercise induced two antagonistic effects masking each other: a thyroid depression due to stress, and a stimulation of the gland attributable to an increased consumption of thyroid hormone as suggested by the rapid disappearance of injected thyroxine from the blood (6). To investigate whether or not such a stimulation takes place, mice were subjected to exercise mild enough to avoid stress.

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METHODS AND RESULTS

Psychological stimulation

A mild stimulus acting over a long period of time may have a definite effect on the thyroid gland, provided it can repeatedly elicit a condition of alertness in spite of frequent repetition over the experimental period. The procedure adopted consisted of providing mice with a new environment each day, by transferring them to different types of cages. Furthermore, in the experience of this laboratory, a change in iodine metabolism is more likely to result in a change of thyroid activity if the iodine intake of the experimental animals is critical. Accordingly, control and experimental animals were subdivided into two groups, given either a low or adequate amount of dietary iodine.

C_3H mice were transferred through 8 cages of various shapes. Since transfers took place once a day, the mice were returned to the same cage every 8 days. Control mice were kept in the same suspended cage throughout the experiment, but they were handled daily. All the animals were housed in groups of 6. Temperature ($80.0 \pm 5°F$) and lighting (12 hours daily) were automatically controlled. Twenty-four mice, 4–5 weeks old, were fed a low iodine diet consisting of 78.85% cornmeal, 10% yeast, 1% calcium carbonate and 0.15% sodium chloride, and containing 3 μg. iodine per 100 gm., while an equal number of animals received the same diet supplemented with sodium iodide to raise the iodine level to 10 μg. per 100 gm. These 2 groups were divided into subgroups of 12 control and 12 test mice.

After 8 weeks, the animals were sacrificed with ether. One thyroid lobe was dissected free and weighed on an analytical balance. The other lobe was fixed in Bouin's fluid, sections were cut at 6 μ, and stained with Masson's trichrome. Thyroid cell height for each animal was measured in 80 cells taken at random. The percentage of follicles depleted of colloid was estimated by examining all follicles in one thyroid section from each animal. The adrenals were weighed after fixation.

In the iodine deficient mice the controls had enlarged thyroids (2.8 mg. /lobe) while the transferred mice had the same thyroid weight (0.8 mg. /lobe) as the iodine supplemented mice (0.7 mg./lobe) (Table 1). The thyroids of mice submitted to a daily change of cage (Table 1) had lower cells and fewer follicles depleted of colloid than those of corresponding control animals regardless of the dietary iodine level. Adrenal weights were identical in transferred and control groups: 2.8 mg. under conditions of low iodine intake and 2.7 mg. with an adequate iodine intake. No changes in body weight were observed in the mice receiving the low iodine diet, while the transferred mice receiving adequate dietary iodine weighed less than their controls (Table 1).

Muscular exercise

To investigate the influence of muscular exercise on thyroid morphology, the mice were placed in a motor driven rotating cage, 3½–4 hours per day, during 8 weeks. The cage had a circumference of 65 cm. and turned at 4½ revolutions per minute, thus enforcing a walking pace. Controls were handled daily. Other conditions were identical with those of the experiment described above.

In the animals fed on the low iodine diet, the exercised mice had a lower thyroid weight, a decreased cell height, and fewer follicles depleted of colloid (Table 2) than the non-exercised controls. In the animals receiving the diet supplemented with iodine, exercise had no significant effect on the

TABLE 1. THE EFFECT OF DAILY TRANSFER OF CAGE ON THE MOUSE THYROID

μg. iodine per 100 gm. diet	Treatment	No. of mice	Body weight, gm.		Left thyroid lobe weight, mg.		Epithelial cell height, μ		% follicles depleted of colloid	
			Mean ±S.E.	P*	Mean ±S.E.	P*	Mean ±S.E.	P*	Mean ±S.E.	P*
3	Controls	9	24.2±1.12	0.8 −0.7	2.8±0.2	<0.001	16.8±0.3	<0.001	91.3±1.9	<0.001
	"Transferred"	8	24.6±0.24		0.8±0.2		9.6±0.3		4.8±0.8	
10	Controls	9	25.4±0.21	0.02−0.01	0.7±0.1	0.5−0.4	11.7±0.3	<0.001	6.0±1.0	0.3−0.2
	"Transferred"	8	23.3±0.21		0.6±0.1		9.3±0.3		4.2±1.3	

* "t" test.

TABLE 2. THE EFFECT OF MILD DAILY MUSCULAR EXERCISE ON THE MOUSE THYROID

μg. iodine per 100 gm. diet	Treatment	No. of mice	Body weight, gm.		Left thyroid lobe weight, mg.		Epithelial cell height, μ		% follicles depleted of colloid	
			Mean ±S.E.	P*	Mean ±S.E.	P*	Mean ±S.E.	P*	Mean ±S.E.	P*
3	Controls	7	21.4±0.26	0.1−0.05	2.8±0.4	0.1−0.05	15.4±0.5	<0.001	94.6± 1.7	<0.001
	Exercised	10	23.4±0.18		1.5±0.5		11.3±0.7		20.3±11.6	
10	Controls	8	24.5±0.25	0.8±0.7	0.9±0.2	0.4±0.3	9.7±0.3	0.2-0.1	4.7± 0.8	0.8−0.7
	Exercised	10	24.9±0.29		1.3±0.2		10.4±0.2		4.9± 1.1	

* "t" test

thyroid gland (Table 2). Adrenal weights were not altered by exercise: 2.7 mg. in both groups of mice receiving the low iodine diet, and 2.3 mg. in both groups receiving the diet supplemented with iodine. No significant changes in body weight were observed.

DISCUSSION

The effect of psychological stimulation and of muscular exercise on the thyroid gland reported in the literature (3, 5) may possibly be due to stress associated with the experimental procedure. The present experiments were an attempt to investigate these stimuli, using procedures avoiding stress.

In fact, the stimuli selected, namely daily changes of housing and mild daily exercise, did not produce the increase in adrenal weight and the decrease in body weight usually associated with prolonged stress, except in one case (Table 1); but in this particular case the thyroid weight and the proportion of follicles depleted of colloid were not changed (Table 1). Thus, it is likely that the lower level of thyroid activity observed in this work is not due to stress.

The possibility that the lower thyroid activity in the stimulated mice be due to a higher iodine intake resulting from increase in food consumption appears very unlikely. While the exercised mice may have ingested more food, the schedule required to refill the food jars suggests that the transferred mice ate not more, and perhaps even less than controls. In addition, an increased food consumption would result in increased metabolism and presumably a higher thyroid hormone consumption. A stimulation of the thyroid would follow, which would counteract the inhibition due to the higher iodine intake.

Thus, it is probable that the thyroid inhibition was produced by the stimuli according to a specific mechanism, perhaps mediated through the hypothalamus (7) and resulting in an inhibition of thyrotrophic hormone secretion.

The finding that psychological stimulation may inhibit the thyroid gland not only for a short time (3), but also over long periods of time (Table 1), fails to substantiate the hypothesis that simple chronic psychological overstimulation is a cause of thyroid hyperfunction, and suggests rather that it may lead to a hypofunction of the gland.

The observation that muscular exercise in the absence of stress also yields evidence of thyroid inhibition does not support the hypothesis that this stimulus had an activating effect on the thyroid gland (5), as might have been expected from the low level of circulating thyroid hormone in the exercised rat (6). On the contrary, muscular exercise prevented the thyroid stimulation in response to iodine deficiency (Table 2), as in the case of chronic psychological stimulation. In fact, during their daily exer-

cise, the mice were in a condition of alertness which may not differ much from that of mice transferred to new cages.

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POTENTIATION OF BIOLOGICAL ACTIVITIES OF STEROIDS BY CARCINOGENIC HYDROCARBONS

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ABSTRACT

Recent studies by Huggins and Pollice (*J. Exp. Med.*, Jan. 1958) showed that a mammary fibroadenoma of rats, refractory to steroid therapy alone, responded in a remarkable fashion to combined administration of 3-methylcholanthrene and androstan-17β-ol-3-one. These results suggested that administration of carcinogenic hydrocarbons may alter sensitivity of certain cells to steroid hormones.

Oral administration of 3-methylcholanthrene, 2-anthramine, benz-α-pyrene, and 7-methylbenzanthracene, all carcinogenic agents, markedly potentiated both the androgenic and myotrophic effects of 17-methyltestosterone. Noncarcinogenic hydrocarbons investigated were nearly inactive in this regard. Oral and direct administration of 3-methylcholanthrene potentiated both the glycogenic and antiphlogistic properties of hydrocortisone. Inactive hydrocarbons, anthracene and phenanthrene, failed to induce this type of phenomenon.

Both *in vivo* and *in vitro* studies indicate that the ability of carcinogenic hydrocarbons to potentiate biological effects of steroids is due to a direct effect of carcinogenic agent at the cellular level and not to a decreased rate of steroid metabolism by the liver.

PREVIOUS studies by other investigators (1, 2) have suggested that local implantation of certain carcinogenic hydrocarbons antagonizes or inhibits the biological response of specific target organs to androgens, estrogens, and progestational agents. Huggins and Pollice (3) recently reported that an experimental mammary fibroadenoma, refractory to steroid therapy alone, showed a marked retardation of growth when androstane-17β-ol-3-one and 3-methylcholanthrene were administered together. When administered alone, neither compound induced a marked degree of inhibition. These results led to an hypothesis that carcinogenic agents may alter the response of certain cells to steroid hormones and that systemic administration of carcinogenic hydrocarbons may produce different effects from those observed following local implantation. The experiments described here were designed to determine the influence of carcinogenic hydrocarbons on biological effects produced by androgens and andrenocortical steroids.

METHODS

The glycogenic effect of hydrocortisone was determined by analysis of liver glycogen deposition in fasted, adrenalectomized rats (4). The seminal vesicle weight of the castrate

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rat was used as an index of androgenic activity of steroids and the levator ani muscle weight as an index of myotrophic activity (5, 6, 7). The ability of hydrocortisone to inhibit inflammation was determined by use of the croton oil-induced granuloma pouch (8). Quantitative methods for determination of steroid metabolism rates by isolated microsome-supernatant fractions of rat liver have been previously described (9). Protein determinations on isolated microsome-supernatant fractions of rat liver were performed according to previously described methods (10). Steroids and carcinogenic agents were administered subcutaneously in 0.2 ml. of carboxymethylcellulose (CMC) or orally as finely dispersed suspensions in 0.5 ml. of cottonseed oil. Direct injection of 3-methylcholanthrene into the granuloma pouch was made by first solubilizing the desired amount of material in 0.2 cc. of 50% ethanol:cottonseed oil. This material was injected into the pouch only if it was found to be in solution. Intact animals received tap water and Purina laboratory chow *ad libitum*. Adrenalectomized rats received 0.9% NaCl in addition to the stock diet.

RESULTS

1. *Potentiation by 3-Methylcholanthrene of Glycogenic and Antiphlogistic Activities of Hydrocortisone*

When hydrocortisone was administered subcutaneously in increasing amounts, and 3-methylcholanthrene orally, it was evident that the carcinogenic agent caused a significant increase in the glycogenic activity of this hormone (Fig. 1). A greater response was elicited in the presence of car-

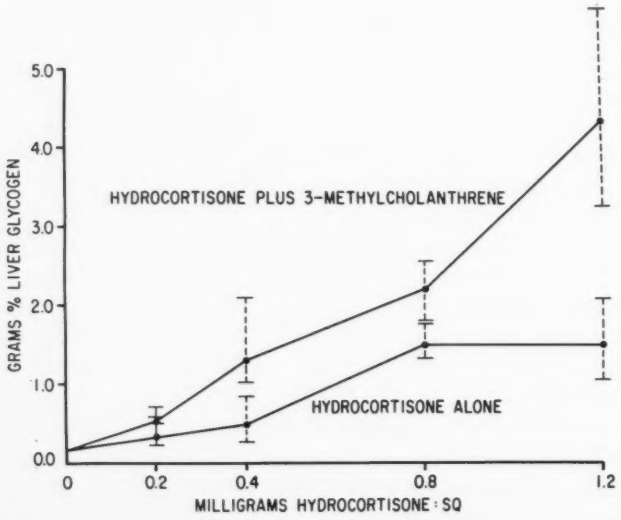

FIG. 1. Effect of oral administration of 3-methylcholanthrene on liver glycogen deposition activity of hydrocortisone. *Conditions:* One week post-adrenalectomized male rats. 3-Methylcholanthrene (2 mg.) administered orally for 5 days. On day 4 hydrocortisone administered subcutaneously at beginning of 24 hour fast; administered again on day 5 with 3-methylcholanthrene. Livers removed 7 hours later. Each point is the mean of 5 rats and the bars indicate variation from the mean.

cinogenic hydrocarbon at doses of hydrocortisone (0.8–1.2 mg.) where the liver glycogen response begins to *plateau*. That is, in the presence of 3-methylcholanthrene, the effect of hydrocortisone is greater than can be produced by the administration of additional hormone. Anthracene and phenanthrene, hydrocarbons inactive as carcinogenic agents, administered under similar circumstances, failed to produce potentiation of the glycogenic effects of hydrocortisone (Table 1).

Potentiation of glycogenic effects of hydrocortisone could result either from a direct action of carcinogenic agent on cells or tissues where steroids exert their metabolic effects, or it may result from a decreased ability of the liver to metabolize steroids. It has been demonstrated previously (9) that glycogenic activity of certain synthetic steroids is inversely related

TABLE 1. EFFECT OF ORAL ADMINISTRATION OF PHENANTHRENE AND ANTHRACENE ON GLYCOGENIC ACTIVITY OF HYDROCORTISONE

Compound	Amount	Route	No. animals	Grams % liver glycogen
A. Hydrocortisone	1.2 mg.	SC	5	2.60
B. Phenanthrene	4.0 mg.	Oral	5	0.13
C. Anthracene	4.0 mg.	Oral	5	0.16
D. Untreated	—	—	10	0.22
E. A+C	—	—	5	2.44
F. A+B	—	—	5	2.53

Conditions: One-week post-adrenalectomized rats. Phenanthrene and anthracene (4.0 mg.) administered orally for 5 days. On day 4 hydrocortisone administered SC at beginning of 24-hour fast; administered again 24 hours later, along with anthracene and phenanthrene. Livers removed for analysis of liver glycogen 7 hours later.

to their rates of metabolism by the liver. Therefore, if oral administration of 3-methylcholanthrene prevented inactivation of hydrocortisone, it might be reflected by an apparent increase in biological activity of the latter.

When varying amounts of 3-methylcholanthrene were added to isolated microsome-supernatant fractions of rat liver, it was found to be inactive with regard to its ability to influence the rate of hydrocortisone metabolism (Fig. 2). Under similar circumstances, direct addition of testosterone decreased the ability of liver enzyme systems to metabolize hydrocortisone. These latter observations will be the subject of a later report on *in vivo* and *in vitro* steroid hormone interrelationships (11).

Oral administration of 2 mg. of 3-methylcholanthrene daily for seven days, followed by removal of livers and analysis of hydrocortisone metabolism rates, indicated that 3-methylcholanthrene-treated livers actually metabolized hydrocortisone at faster rates than livers from pair-fed control animals (Fig. 3).

Attention was directed to a consideration of the ability of 3-methylcholanthrene to potentiate the antiphlogistic properties of hydrocortisone using croton oil inflammation as an end-point (8). Both the carcinogen

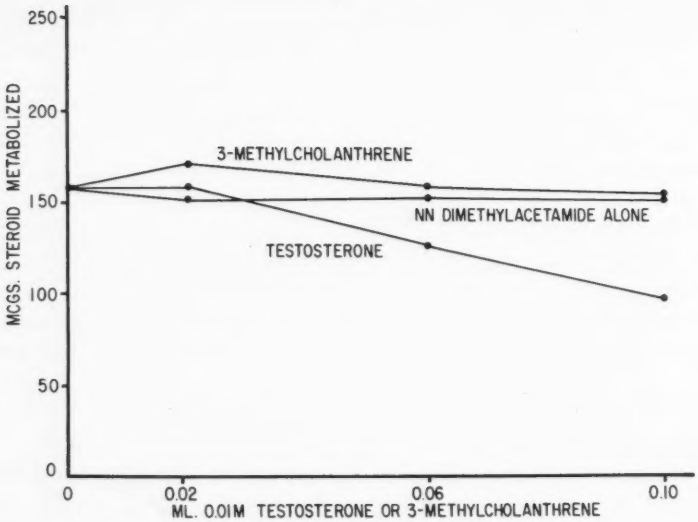

FIG. 2. Effect of testosterone and 3-methylcholanthrene on rate of reduction of hydrocortisone at C-20 ketone position by rat liver microsome-supernatant fractions. *Conditions:* Described in previous reports (9). Steroids and 3-methylcholanthrene dissolved in N'N'-dimethylacetamide.

and hydrocortisone were injected directly into the granuloma pouch. Results of these experiments (Figs. 4 and 5) show that direct administration of 3-methylcholanthrene into the granuloma pouch significantly enhances the anti-inflammatory response to hydrocortisone in both intact and adrenalectomized rats.

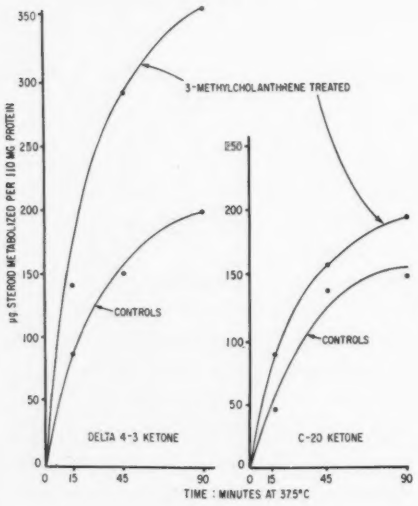

FIG. 3. Comparison 3-methylcholanthrene treated liver versus untreated liver on metabolism of hydrocortisone by isolated liver enzyme systems. Each point is the mean of duplicate determinations on duplicate samples from livers of 3 rats.

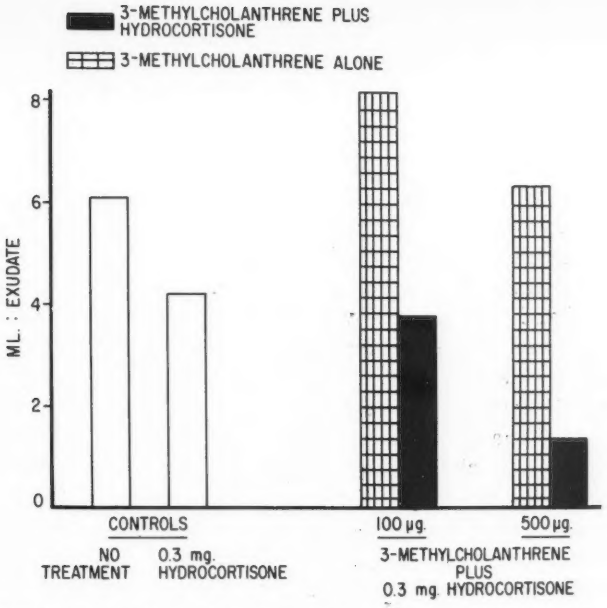

FIG. 4. Local potentiation of hydrocortisone by 3-methylcholanthrene on the granuloma pouch of Adrenalectomized rats. *Conditions:* 25 ml. air and 0.5 ml. 1% croton oil into pouch on day 1. Hydrocortisone and 3-methylcholanthrene administration started on day 2. Animals autopsied on day 7. Each bar is the mean of 5 rats.

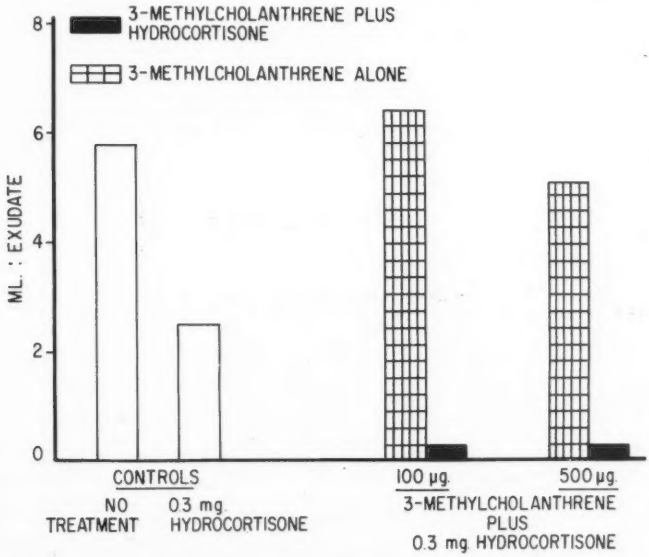

FIG. 5. Local potentiation of hydrocortisone by 3-methylcholanthrene on the granuloma pouch of intact rats. *Conditions:* 25 ml. air and 0.5 ml. 1% croton oil into pouch on day 1. Hydrocortisone and 3-methylcholanthrene administration started on day 2. Animals autopsied on day 7. Each bar is the mean of 5 rats.

2. *Effects of Various Carcinogenic Hydrocarbons on Androgenic and Myotrophic Activities of 17-Methyltestosterone*

When 3-methylcholanthrene and 17-methyltestosterone were administered orally to castrate rats, the carcinogen caused a marked potentiation of androgenic effects of the latter (Fig. 6). Relatively small amounts of 3-methylcholanthrene (0.5 mg./day) produced a maximum response.

Oral administration of 2 mg. of 3-methylcholanthrene, in the presence of varying amounts of 17-methyltestosterone, resulted in significant enhancement of androgenic activity of the latter at all dose levels (Fig. 6A). Animals employed in these latter studies were 27 days older than animals used for previous studies and were less sensitive to the action of orally administered 17-methyltestosterone.

As a result of the above investigations, it became of interest to determine if oral 3-methylcholanthrene would induce an irreversible change in cells causing them to become more sensitive to the androgenic effects of 17-methyltestosterone. 3-Methylcholanthrene (2 mg.) was administered orally for 10 days to 55-day old castrate male rats; animals were subsequently allowed to remain untreated for an additional 10 days; 5 mg. of 17-methyltestosterone were thereupon administered for an additional 10 days. Examination of Figure 6B reveals that potentiation of androgenic effects of 17-methyltestosterone did not persist following the 10-day withdrawal period. Thus, the influence of 3-methylcholanthrene on augmentation of biological response to 17-methyltestosterone is not irreversible, but exists only during administration of the carcinogen.

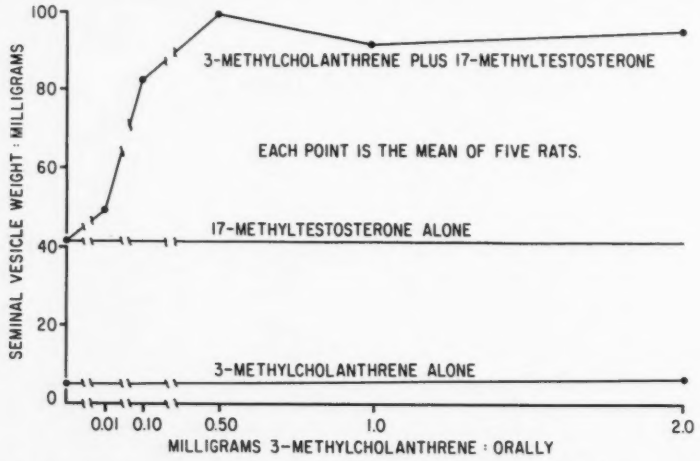

FIG. 6. Effect of orally administered 3-methylcholanthrene on response of seminal vesicle to 17-methyltestosterone. *Conditions:* Male rats (26–28 days old) castrated on day 1. 3-Methylcholanthrene administered orally in varying amounts for 8 days. 17-Methyltestosterone administered orally for 8 days. Animals autopsied on the 9th day.

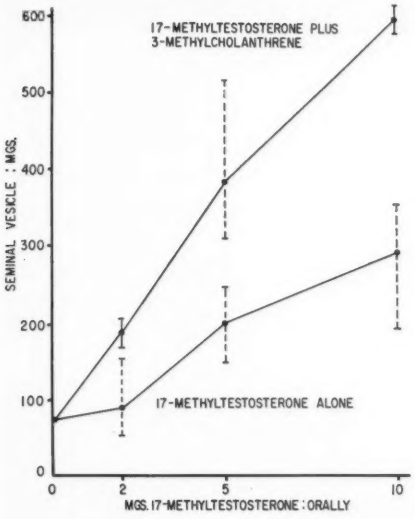

FIG. 6A. Effect of 3-methylcholanthrene administration on seminal vesicle response to 17-methyltestosterone. *Conditions:* 55-Day-old male rats castrated on day 1. Compounds administered orally starting on day 2 and continued for 10 days. Autopsy on day 11. Each point is the mean of 5 rats. Bars represent variation from the mean.

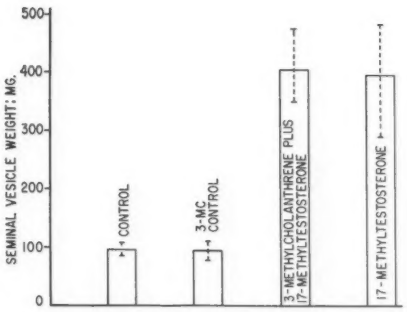

FIG. 6B. Effect of prior administration of 3-methylcholanthrene (3-MC) on response of seminal vesicle to 17-methyltestosterone. *Conditions:* 55-Day-old male rats castrated on day 1. 3-Methylcholanthrene administered on day 2 and continued for 10 days. 17-Methyltestosterone administered 10 days following cessation of 3-methylcholanthrene administration and continued for 10 days. Autopsy 30 days following initial injection with 3-methylcholanthrene. Bars indicate the mean variation: 5 rats per group.

Administration of 3-methylcholanthrene (2 mg./day for 20 days) to intact male rats (20) did not augment the action of endogenously secreted androgenic hormones on the seminal vesicle. Relative growth rates of seminal vesicles for both control-treated and 3-methylcholanthrene-treated animals were identical.

It became of interest to determine if 3-methylcholanthrene administration would result in augmentation of 17-methyltestosterone at dose levels of the latter where it no longer exerts an increased biological effect. Varying amounts of 17-methyltestosterone were administered orally with 3-methylcholanthrene (2 mg.). Examination of Table 2 reveals that, although 3-methylcholanthrene administration induces the expected augmentation of biological response to 17-methyltestosterone, it fails to do so at dose levels of the latter which alone produce a maximal biological effect.

When 3-methylcholanthrene and testosterone propionate were both administered subcutaneously, it was discovered that the hydrocarbon significantly potentiated the "androgenic" effects of this steroid (Fig. 7). However, the amount of augmentation produced by this route of administration was considerably less than following oral administration. These

TABLE 2. EFFECT OF 3-METHYLCHOLANTHRENE ADMINISTRATION ON RESPONSE OF LEVATOR ANI MUSCLES AND SEMINAL VESICLES OF CASTRATE MALE RATS TO 17-METHYLTESTOSTERONE

	Treatment	No. rats	Dose/Day (orally)	Seminal vesicle weight (mg.)	Levator ani weight (mg.)
A	None	5	—	7	30
B	3-Methylcholanthrene	5	20 mg./kg.	10	28
C	17-Methyltestosterone	5	10 mg./kg.	30	42
D	17-Methyltestosterone	5	20 mg./kg.	59	60
E	17-Methyltestosterone	5	40 mg./kg.	78	61
F	17-Methyltestosterone	5	80 mg./kg.	131	77
	B+C	5		58	59
	B+D	5		83	57
	B+E	5		116	72
	B+F	5		130	78

results may be due to inadequate absorption of 3-methylcholanthrene, since nodules containing injected material were found at autopsy. The effects of testosterone propionate on the levator ani were not significantly augmented by simultaneous administration of 3-methylcholanthrene.

Oral administration of 2-anthramine, benz-α-pyrene and 7-methyl-

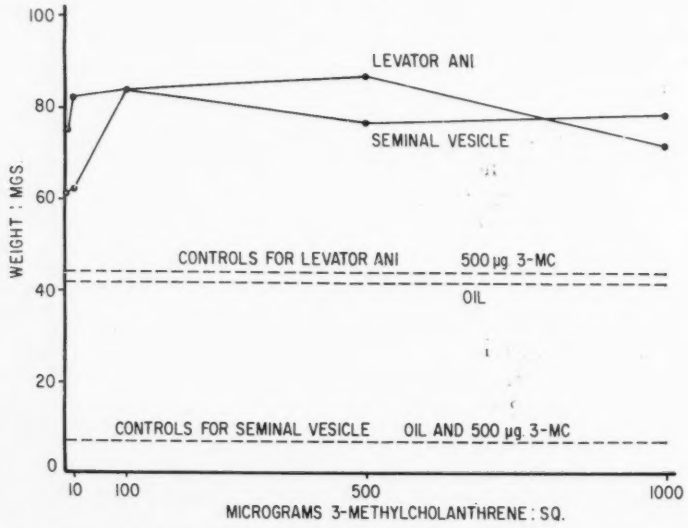

FIG. 7. Effect of 3-methylcholanthrene on the seminal vesicle and levator ani response to subcutaneously administered testosterone propionate. *Conditions:* Castrate rats 26–28 days old. Injections started on day 1 and continued for 20 days. Testosterone propionate 25 µg per day per rat. Animals autopsied on day 21. Each point is the mean of 6 rats.

benzanthracene, all carcinogenic agents, potentiated the androgenic and myotrophic effects of 17-methyltestosterone, while noncarcinogenic agents, anthracene, phenanthrene, anthrone and anthroquinone, failed to potentiate significantly the biological effects of testosterone propionate or of 17-methyltestosterone (Table 3). Deoxycholic, methyldeoxycholic, and dehydrocholic acids were used as additional controls for the above studies and were also found to be devoid of significant activity (Table 3).

TABLE 3. EFFECT OF BILE ACIDS, CARCINOGENIC HYDROCARBONS, AND OTHER AGENTS ON THE RESPONSE OF THE SEMINAL VESICLE AND LEVATOR ANI TO TESTOSTERONE DERIVATIVES

Compound	Dose	Route	Seminal vesicle (wt., mg.)	Levator ani (wt., mg.)
1. Methyltestosterone	2.0 mg./day	Oral	30.0	57.0
2. 2-Anthramine	0.5 mg./day	Oral	6.0	30.0
Nos. 1 & 2			77.0	75.0
3. Benz-α-pyrene	0.5 mg./day	Oral	7.0	27.0
Nos. 1 & 3			66.0	78.0
4. 7-Methylbenzanthracene	1.0 mg./day	Oral	7.0	32.0
Nos. 1 & 4			79.0	72.0
5. Anthrone	2.0 mg./day	Oral	6.0	30.0
Nos. 1 & 5			39.0	61.0
6. Anthraquinone	5.0 mg./day	Oral	6.0	30.0
Nos. 1 & 6			38.0	49.0
1. Testosterone propionate	25 μg./day	SC	61.0	74.0
2. Anthracene	100 μg./day	SC	7.0	50.0
Nos. 1 & 2			60.0	75.0
3. Phenanthrene	100 μg./day	SC	6.0	56.0
Nos. 1 & 3			54.0	72.0
Cottonseed oil	0.2 ml./day	Oral	7.0	31.0
1. Methyltestosterone	2.0 mg./day	Oral	27.0	49.0
2. Deoxycholic acid	5.0 mg./day	Oral	7.0	33.0
Nos. 1 & 2			38.0	52.0
3. Deoxycholic acid (methyl ester)	5.0 mg./day	Oral	5.0	29.0
Nos. 1 & 3			33.0	44.0
4. Dehydrocholic acid	5.0 mg./day	Oral	5.0	30.0
Nos. 1 & 4			33.0	49.0

Conditions: Six animals per group. Castrate male rats, 26–28 days old. Compounds administered orally for 9 days or subcutaneously for 20 days. On days 10 and 21 animals sacrificed and levator ani muscle and seminal vesicles weighed on precision balance.

DISCUSSION

Studies have been presented to show that oral administration of the carcinogenic hydrocarbons, 3-methylcholanthrene, 2-anthramine, benz-α-pyrene, and 7-methylbenzanthracene, results in marked potentiation of the androgenic or myotrophic effects of 17-methyltestosterone. 3-Methylcholanthrene also potentiates the biological action of hydrocortisone on two separate end points—liver glycogen and granuloma pouch. Observations that four unrelated organs, the levator ani muscle, seminal vesicle, liver and granuloma pouch, respond in similar fashion to administered carcinogenic agents indicate a type of cellular activity which may be un-

related to the type of organ response ordinarily associated with specific activities. That is, carcinogenic hydrocarbons may elicit fundamental cellular changes in all organs and tissues. It appears logical to suppose that carcinogenic hydrocarbons are involved in a generalized cellular alteration, permitting cells to become more sensitive to naturally-occurring agents. Such ideas are merely speculative, but it may be postulated that tumorigenic hydrocarbons are not cancer-inducing *per se*, but merely alter cells in a manner which depresses normal control mechanisms, thus allowing cells to lose their regulatory capacity toward specific agents.

The increased sensitivity of cells to naturally-occurring agents, following oral administration of carcinogenic compounds, may be reflected by systems other than those responsive to steroid hormones. The above experiments suggest a technological mode of attack on the problem of determining the influence of various etiological factors concerned with the tumor process. Knowledge of a useful fundamental character should result from studies designed to determine the types and incidence of tumors following oral administration of carcinogenic hydrocarbons and various agents such as viruses and hormones.

The results reported here are difficult to reconcile with those described by Jackson and Robson (1) who applied 3-methylcholanthrene directly to target organs and reported that this agent *inhibited* the response of specific end organs to both estrogens and androgens. The only control implantation was that which employed cholesterol; not, as in our study, inactive, but related, hydrocarbons. It is important in situations of this type to define the specificity of the material being investigated. Direct addition of any substance to a tissue may produce results not seen when the substance reaches the tissue by normal channels—especially if the substance is as insoluble as are carcinogenic hydrocarbons. Inhibition of a response under such conditions may be the result of nonspecific physical factors and not a reflection of specific biological effects. On the other hand, our own results with carcinogenic agents could be attributed to a bioconversion of carcinogenic hydrocarbons in the liver or other tissues to agents which themselves are responsible for the potentiation of hydrocortisone and 17-methyltestosterone.

On the basis of the existing data, it may be postulated that oral administration of carcinogenic hydrocarbons "sensitizes" cells to the action of a number of steroids at different levels of tissue organization. It may also be postulated that oral administration of carcinogenic agents may "sensitize" cells to a wide variety of agents. For example, Castellano and D'Amour (12) found that administration of stilbestrol increased the incidence of u'erine tumors following implantation of 3-methylcholanthrene. Under certain conditions, the estrogens themselves are carcinogenic (13). If 3-methylcholanthrene potentiates the biological action of estrogens, these

latter results are readily interpreted on the basis of observations presented in this communication.

That carcinogenic hydrocarbons may "sensitize" cells to the action of other agents is readily apparent from work reported by Berenblum (14). This investigator showed that croton oil applied alone to the skin of mice induces hyperplasia, but normally no tumors develop during the animals' lifetime. If dimethylbenzanthracene is applied alone, hyperplasia develops and, after a prolonged period, some tumors develop. If both croton oil and dimethylbenzanthracene are applied together, tumors appear more rapidly and with greater frequency than when dimethylbenzanthracene alone is applied. Even more striking is the fact that if just one application of dimethylbenzanthracene is made, tumors appear after a protracted period. But if, after the application of dimethylbenzanthracene, croton oil is applied, tumors develop more rapidly, and there is a strong tendency for tumors to appear at a standard time following croton oil application rather than following application of dimethylbenzanthracene. It appears evident that dimethylbenzanthracene causes some irreversible change in cells which remains latent until the application of croton oil. It is interesting in this regard that croton oil-induced inflammation, used in our experiments, and which is inhibited by hydrocortisone, is inhibited even more significantly in the presence of 3-methylcholanthrene. These appear to be actions of opposite extreme attributable to a "conditioning" response brought about by a carcinogenic hydrocarbon. It may well be that carcinogenic agents will produce effects which are either inhibitory or stimulatory depending upon additional circumstances imposed during their administration. As a matter of interest, as well as some practical importance, it may be visualized that the action of carcinogenic hydrocarbons is involved with a type of cellular alteration which exists for prolonged periods following their administration. This type of alteration may never become apparent until the necessary stimulus for cellular proliferation, virus, hormone or foreign agent, is permitted contact with the altered cell. This type of phenomenon may explain the appearance of tumors months or even years after initial contact with carcinogenic agents.

As a matter of theoretical and practical importance it becomes necessary to determine if chemical or physiological means exist which, when applied, will prevent some of the cellular "conditioning" responses to carcinogenic agents such as those illustrated herein. Such information, if it exists experimentally, should be carried over to prophylactic treatment of experimental animals known to have a high incidence of spontaneous tumors. Such efforts will determine if the incidence of tumors can be decreased or completely abolished. Another avenue of approach is one which attempts to more clearly define some of the etiological factors in tumor formation. Combined administration of carcinogenic compounds and naturally-occur-

ring agents such as viruses and hormones should result in development of tumors at a faster than normal rate as well as the appearance of newer types of tumors.

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A METHOD FOR THE EVALUATION OF ADRENOCORTOCOTROPIC HORMONE SUPPRESSING ACTION OF CORTICOIDS[1]

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ABSTRACT

A simple, reproducible method employing the rat is described which is suitable for the evaluation of compounds as to their adrenocorticotropic hormone suppressing action. The endpoint is the adrenal corticosterone content which reflects the concentration of circulating ACTH. With excessive doses of cortisone acetate a 93% decrease in adrenal corticosterone was found. The relative potency of a compound could be determined with a standard error of ±18% by using 20 rats for the standard and 20 rats for the unknown.

IT IS well known that the administration of various corticosteroids causes a decreased elaboration of adrenocorticotropin (ACTH) from the pituitary of various animals. This, in turn, leads to a decreased biosynthesis and release of adrenocorticoids as is reflected also in the hypotrophic and subfunctional state of the adrenal gland. Holzbauer (1) recently presented good evidence showing that the corticosterone stores of the rat adrenal gland reflect the secretion rate of corticosteroid. This in turn reflects the level of circulating ACTH since high or low corticosterone values are obtained when conditions leading to large or small amounts of circulating ACTH respectively are brought about. Thus, any condition which would interfere with the elaboration of ACTH would also be expected to be reflected in the amount of corticosterone in the rat adrenal gland. Silber and Busch (2) have found that the adrenals of rats 2 weeks after hypophysectomy contained 0.04 μg. corticosterone per adrenal as compared to values of 0.30–0.50 μg. for normal adrenals. The same values as in the adrenals of hypophysectomized animals were found after treatment with cortisone acetate at a level of 6 mg./day subcutaneously for 3 weeks (2). Whereas Sweat (3) used ethanolic solutions of steroids for producing fluorescence in sulfuric acid and Peterson (4) later modified this method, the method used in the present paper (2) consists of extracting the tissue steroid directly into methylene dichloride or chloroform. The extracted steroid is then allowed to react with sulfuric acid by adding the latter directly to the methylene dichloride. This communication demon-

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strates that this simple fluorometric method for the direct determination of adrenal corticosterone can be used as the basis of a satisfactory bioassay for the evaluation of the ACTH suppressing action of corticoids.

MATERIALS AND METHODS

Animals and protocols

The steroids suspended in Special Formula No. 17874[2] were injected subcutaneously into male albino rats weighing 100–175 gm. The daily dose was contained in 0.25 ml. and control rats received only the vehicle. On the day following the last injection each rat was treated with nembutal, taking every precaution to handle the animals as gently as possible (1) and the adrenals removed, cleaned of adherent fat, and weighed.

Fluorometric method

The method of extracting the steroid from the tissue and development of the fluorescence in the extracts of tissue is given elsewhere (2). This method has formed the basis also for the measurement of corticosterone in rat plasma by Guillemin *et al.* (5). The corticosterone content of the rat adrenals was determined by measuring the fluorescence emitted with the Aminco-Bowman Spectrophotofluorometer. The activation monochromator was set at λ max 470 mμ and the fluorescence monochromator at λ max 525 mμ. These are the uncorrected fluorometric maxima. Samples were read on the photomultiplier microphotometer with the sensitivity knob set at the maximum position and the meter multiplier at the 1.0 position. The slit arrangement was No. 5. The fluorescence was proportional to the amount of corticosterone used (0.02–0.08 μg.) for the standard curve and the photomultiplier reading was in the neighborhood of 35 for the 0.08 μg. concentration with the settings as stated. A standard curve was run with each experiment. Figure 1 shows the data of recovery experiments plotted graphically wherein several concentrations of corticosterone were added to a fixed weight of adrenal tissue (10 mg.). The reliability of the method was tested further by running converse experiments wherein the amount of adrenal tissue was varied but the amount of corticosterone added to it kept constant. Figure 2 shows that additions of 0.04 μg. corticosterone (Experiments 1 and 2) and 0.08 μg. (Experiments 3 and 4) to varying amounts of adrenal tissue (1.25 ml. of tissue aliquot = 10 mg. of adrenal tissue) does not interfere with the extraction of corticosterone or its recovery from the tissue. Since the experimental curves are linear and their slopes proportional to the amount of tissue used, after subtracting the value found for the added corticosterone, this indicates that the quenching phenomena often observed in fluorometric determinations are absent. Table 1 gives data showing the specificity of the method by indicating the very low apparent concentrations of corticosterone-like compound found in extra adrenal tissue.

RESULTS AND DISCUSSION

Table 2 shows the concentration of corticosterone[3] found in the adrenals of control rats and those treated for a 21-day period with 9α-fluorocorti-

[2] Special Formula No. 17874 is a product obtained from the Upjohn Co., Kalamazoo, Michigan. It is a mixture of carboxymethycellulose, polysorbate 80, sodium chloride and benzyl alcohol in water for injection.

[3] It is assumed that the steroid giving rise to fluorescence is corticosterone since very small amounts of other steroids are found in the rat adrenal (1). This also excludes aldosterone which is not detected by procedure (6) although this steroid is released in a ratio of about 10/1 of corticosterone to aldosterone in the incubation medium of rat adrenals incubated in Krebs-Ringer bicarbonate-glucose buffer (7).

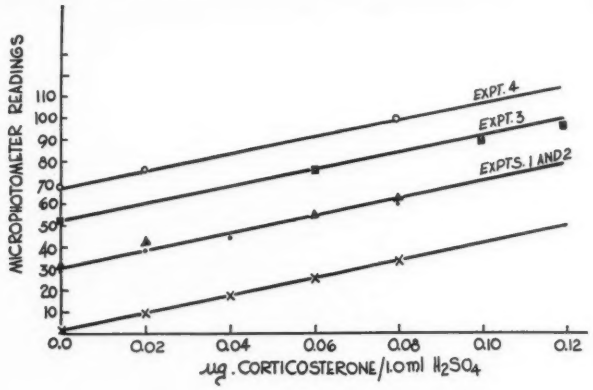

FIG. 1. Recovery Experiment. ×——× Standard curve for corticosterone 0.02–0.08 μg. Other curves represent those obtained when various amounts of corticosterone (0.02–0.12 μg.) are added to 10 mg. of rat adrenal tissue. The difference between the blank value of the standard curve and the values for the experiments on the ordinate are equal to the readings given by the tissue alone. Markings on the Experiment 1–4 curves (▲, ●, ■, ○) show the additions of corticosterone at the concentration denoted on the abscissa. Parallelism of all curves with that of the standard curve denotes good recovery.

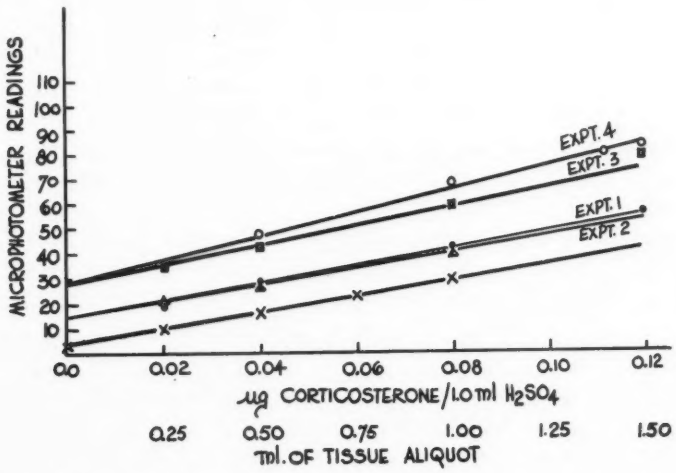

FIG. 2. Modified Recovery Experiment. ×——× Standard curve for corticosterone 0.02–0.08 μg. The double plot on the abscissa denotes the concentration of corticosterone used for the standard curve (μg. corticosterone/1.0 ml. H_2SO_4) and the amount of tissue used in Experiments 1–4 (ml. of tissue aliquot). Markings on the Experiment 1–4 curves (▲, ●, ■, ○) show the amount of tissue aliquot used in each experiment. 0.04 μg. of corticosterone (Experiments 1 and 2) and 0.08 μg. of corticosterone (Experiments 3 and 4) were added to the aliquots of tissue used for analysis. Extrapolation points of the curves of Experiments 1 and 2 on the ordinate when read on the standard curve should read 0.04 μg. corticosterone and by the same token 0.08 μg. for Experiments 3 and 4.

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TABLE 1. APPARENT CORTICOSTERONE CONCENTRATION IN DIFFERENT TISSUES OF THE RAT

Tissue	Wet weight, mg.	Total corticosterone, μg.
Liver	9.6	0.010
	10.4	0.010
	20.3	0.014
	20.0	0.016
	40.0	0.016
	41.0	0.016
Kidney	11.4	0.010
	10.0	0.010
	20.6	0.016
	22.0	0.015
	41.0	0.015
	42.0	0.019
Brain	11.4	0.017
	11.2	0.016
	20.8	0.021
	21.2	0.021
	41.0	0.029
	41.0	0.029

sone acetate and cortisone acetate respectively. Nine of the ten rats injected with 9α-fluorocortisone acetate did not survive the injection period while two died in the group receiving the cortisone acetate. The over-all results indicate clearly the greatly decreased content of corticosterone in the rat adrenals as a result of steroid administration. Cortisone acetate caused a 93% decrease in adrenal corticosterone and a 60% decrease in adrenal weight reduction and essentially confirms the findings of Silber and Busch (2). In the single survivor of 9α-fluorocortisone acetate treatment a similarly low adrenal corticosterone was found. Analysis of weight and adrenal corticosterone content of the left and right glands revealed no significant difference (Table 2).

Table 3 shows the results obtained when cortisol was injected over a fairly wide range of concentrations in seven trials. The minimum dosage level at which cortisol caused a significant decrease in adrenal cortico-

TABLE 2. INFLUENCE OF CORTISONE ACETATE AND 9α-FLUOROCORTISONE ON THE CORTICOSTERONE CONTENT OF RAT ADRENALS
(Treatment period was 21 days)

Material administered	Total dose, mg.	No. of rats	Mean adrenal weight, mg. ±S.E.		Mean corticosterone in gland, μg. ±S.E.	
			Left adrenal	Right adrenal	Left adrenal	Right adrenal
0	0	10	18.1±1.0	19.9±0.9	1.33±0.13	1.31±0.14
Cortisone Acetate	48.3	18*	8.6±1.0	8.7±0.7	0.09±0.008	0.10±0.120
9α-Fluorocortisone Acetate	21	1†	8.5	8.0	0.09	0.09

* 2 animals died during experimental period.
† 9 animals died during experimental period.

TABLE 3. INFLUENCE OF CORTISOL ON THE CORTICOSTERONE CONTENT OF RAT ADRENALS
(Treatment period was 7 days)

Material administered	Total dose, mg.	No. of rats	Mean corticosterone per adrenal, μg. ±S.E.
0	0	12	0.68 ±0.03
Cortisol	0.70	12	0.77 ±0.09
	2.10	11	0.72 ±0.06
	6.30	11	0.45 ±0.03
0	0	12	0.80 ±0.06
Cortisol	0.70	12	0.88 ±0.10
	2.10	12	0.78 ±0.08
	6.30	11	0.41 ±0.04
0	0	12	0.76 ±0.07
Cortisol	6.30	9	0.48 ±0.06
	12.60	10	0.26 ±0.02
	18.90	11	0.26 ±0.01
0	0	12	0.92 ±0.08
Cortisol	6.30	12	0.52 ±0.05
	12.60	12	0.29 ±0.02
	18.90	12	0.27 ±0.03
0	0	6	0.83 ±0.19
Cortisol	3.15	12	0.71 ±0.07
	6.30	12	0.55 ±0.07
	12.60	12	0.34 ±0.01
0	0	11	0.82 ±0.05
Cortisol	3.15	12	0.68 ±0.04
	6.30	12	0.38 ±0.03
	12.60	12	0.27 ±0.01
0	0	12	1.13 ±0.07
Cortisol	3.15	12	0.82 ±0.05
	6.30	12	0.74 ±0.07
	12.60	12	0.36 ±0.02

sterone was found to be 3.15 mg. per 7 days, while 12.60 mg. per 7 days caused a maximum decrease. The decrease in adrenal corticosterone was proportional to the amount of cortisol administered between the limits of 3.15 to 12.6 mg. per animal over the 7-day period. In six trials slopes rang-

TABLE 4. INDEX OF PRECISION OF ASSAY USING CORTISOL

N	Total dose levels, mg.	Slope b	Index of precision λ
22	2.1; 6.3	−0.572	0.260
23	2.1; 6.3	−0.711	0.296
19	6.3; 12.6	−0.758	0.158
24	6.3; 12.6	−0.773	0.149
36	3.15; 6.3; 12.6	−0.615	0.276
36	3.15; 6.3; 12.6	−0.764	0.222

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ing from −0.572 to −0.773 were found with a mean of −0.699. The index of precision (λ) varied from 0.149 to 0.296 for these trials with a mean of 0.227 (Table 4). On the basis of these calculations it appears that the relative potency of a compound could be determined in relation to cortisol with a standard error of ±18% by using a total of 40 animals, 20 on the unknown and 20 on the standard.

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ADRENAL CORTICOID ACTIVITIES OF 9α-FLUORO-11β,16α,17α,21-TETRAHYDROXY-1,4-PREGNADIENE-3,20-DIONE[1,2,3]

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ABSTRACT

Triamcinolone (9α-fluoro-11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione) was studied in rats to assess its adrenocortical activities using the following assays: electrolyte excretion, liver glycogen deposition, growth-survival and anti-inflammatory activity (asbestos pellet technique).

Subcutaneously administered triamcinolone was 3 to 12 times more potent than prednisolone and 10 to 40 times more active than hydrocortisone in liver glycogen deposition tests. On oral assay, the relative potency of triamcinolone was higher.

Triamcinolone proved to be 5 to 10 times more active than hydrocortisone acetate in the anti-inflammatory assay.

Triamcinolone was more efficacious than hydrocortisone and at least as effective as DOC in maintaining the life of immature, male, adrenalectomized rats. Although survival time was prolonged, there was a suppression of growth when compared with the DOC-treated rats.

Triamcinolone was found to be a potent diuretic and natriuretic steroid.

EFFORTS to synthesize useful therapeutic analogs of hydrocortisone and cortisone have resulted in numerous steroids possessing pronounced mineralo- and glucocorticoid activities. Structural modifications of hydrocortisone which enhanced glucocorticoid activity include halogenation at carbon 9 (1, 2), introduction of a double bond between carbon 1 and 2 (3–6), and methylation at either carbon 2 (7, 8) or carbon 6 (9).

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[1] These experiments were conducted in the Pharmacological Research Department (Dr. Earl H. Dearborn, Department Head) of the Experimental Therapeutics Research Section (Dr. John T. Litchfield, Jr., Director) Research Division, American Cyanamid Company, Pearl River, New York.

[2] This work was initiated through the efforts of Drs. Fred I. Dessau and Paul H. Bell. The authors wish to express their gratitude to these investigators for their aid and advice in pursuance of this study. The authors are also indebted to Dr. Seymour Bernstein and associates for the generous supplies of triamcinolone and to Messrs. C. W. Dunnett and J. D. Haynes for the statistical evaluation.

[3] A preliminary report of this work was presented before the Society of Pharmacology and Experimental Therapeutics, Baltimore, Md., 1957. *J. Pharmacol. and Exper. Therap.* **122,** 60A, 1958.

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Further augmentation of activity was accomplished by combinations of two or more of the above molecular modifications (8, 10, 11); however, an increase in electrolyte activity (sodium retention-potassium excretion) is associated with innovations such as 9α-halogenation and 2α-methylation of hydrocortisone. Prednisolone (Δ^1-hydrocortisone) reported to be three times as potent as hydrocortisone in animal assays, "failed to produce sodium retention in adrenalectomized rats" (6), whereas 6α methyl-Δ^1-hydrocortisone, also a potent glucocorticoid, was diuretic and natriuretic (10).

In the quest for further interesting chemical substitutions, investigations in the biosynthesis of adrenal corticoids by a variety of species have suggested the presence of compounds more polar than hydrocortisone (12). Considerable interest has been shown in corticoids possessing additional hydroxyl groups at positions 6 and/or 16 (13, 14). In a review article Bernstein (15), presents a list of some fourteen steroids, hydroxylated in the 16 position, which have been isolated from urine and characterized.

16α-Hydroxy desoxycorticosterone was first prepared in 1953 by Hirschmann, Hirschmann, and Farrell (16), who observed that the "compound failed to demonstrate the high salt retaining activity of electrocortin." Others, who have also reported on this compound, found that sodium-retention activity is absent or weak (17, 18).

In further pursuance of this modification as a means of dissociating the undesirable sodium retentive activity of certain potent glucocorticoids, a number of 16α-hydroxylated compounds have been prepared by Bernstein and his collaborators (19–23). The most active, 9α-fluoro-11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione (triamcinolone) possessed a glucocorticoid potency 3 to 12 times that of prednisolone. Furthermore, triamcinolone exhibited a marked ability to promote water and sodium diureses.

An elucidation of the biological efficacy of this compound in comparison with other steroids is the aim of the experiments reported.

METHODS

Urinary excretion and liver glycogen deposition assay

In this assay, the biological effects of adrenocorticoids on electrolyte excretion and liver glycogen deposition were assayed simultaneously in the same animal.

Steroid suspensions were prepared by grinding the steroids with the vehicle in ground glass homogenizers. The vehicle contained 0.5% carboxymethylcellulose and 0.4% Tween 80 in 0.9% sodium chloride solution. This modification of the medium of Stafford *et al.* (11) excludes benzyl alcohol.

Adult male rats (Sherman or Wistar strain, 140–170 Gm.) were bilaterally adrenalectomized, and were given 1% sodium chloride in their drinking water and the high protein diet recommended by Olson *et al.* (24). All animals were held for 4 days after adrenalectomy to allow clearance of residual adrenal steroids. On the fourth postoperative day, the animals were weighed and randomly grouped, and the food was removed to insure

maximum depletion of liver glycogen. Twenty-four hours later, the saline was discontinued, and graded doses of the steroids, in 0.2 ml. of vehicle per dose, were injected subcutaneously, or in 0.5 ml. of vehicle per dose, were administered orally by gavage. Control rats received only the carboxymethylcellulose vehicle.

Thirty minutes after the administration of the steroid, the rats were lightly etherized to insure complete emptying of the bladder. They were then injected intraperitoneally with 5 ml. of 0.9% sodium chloride solution, and two rats were placed in each metabolism cage. The urine was collected in graduated cylinders after passing through a glass wool filter placed in the mouth of the funnel to prevent fecal contamination. No food or water was allowed during the assay. Five hours after saline administration, the glass wool filters were squeezed to remove trapped urine and the volume was recorded. The cages and funnels were then rinsed with distilled water until the final volume was 25 ml. The milliequivalents of urinary sodium and potassium per pair of rats were determined by flame photometry.

Seven hours after dosing, all animals were anesthetized with 40 mg. of sodium pentobarbital per kilogram of body weight. The livers were quickly excised and immediately hydrolyzed in 30% potassium hydroxide (Pabst *et al.* (25)). The total liver glycogen was determined by the anthrone method of Seifter *et al.* (26).

The relative potencies of the steroids involved were statistically analyzed by determining the linear regression lines by the method of least squares. Since these lines were not parallel, the relative potencies varied with the response level. The 0.4 and 1.2% liver glycogen points were arbitrarily chosen as the response levels at which the potencies are quoted.

Life maintenance and growth

Immature male rats (40–60 gm.) were bilaterally adrenalectomized and immediately injected subcutaneously with graded doses of the steroids suspended in 0.2 ml. of carboxymethylcellulose vehicle. Both sham-operated (rats whose adrenals were exposed but not removed, under identical operating conditions) and adrenalectomized controls received vehicle only. The injections continued daily for 14 days. The animals were maintained on Purina dog chow and tap water *ad libitum*.

Weights were recorded every 3 days throughout the 28-day period of the assay. Animals were checked daily and any rats surviving to the 28th day, with the exception of those sham-operated, were autopsied and examined for residual adrenal tissue. Those found to have evidence of adrenal tissue were eliminated from the experiment. This experimental procedure was modified from that described by Tolksdorf *et al.* (6).

Anti-inflammatory test

The test system is a modification of Meier's cotton pellet method (27). Female albino rats (100–120 gm.) were anesthetized, and a 30 mg. $\pm$1 mg. asbestos pellet (Seitz S-1, sterilizing) was placed in each of two subcutaneous pockets in the left and right dorsal areas. Twice daily, beginning within two hours postoperatively, groups of 6 rats were dosed subcutaneously with 0.1 ml. of the steroid suspension. The concentrations of the steroid suspensions were such that one half the desired daily dose was contained in 0.1 ml. The control group, consisting of twice as many animals as the test group, was treated twice daily with 0.1 ml. of vehicle. Treatment was continued for 5 days. On the 6th day, the rats were sacrificed and the pellets, with accompanying granuloma tissue, were excised and weighed.

Statistical analyses similar to those employed in the liver glycogen studies were used to determine the relative potencies. Here again, the regression lines were not parallel so the 600 and 1000 mg. pellet weights were chosen as the response levels for comparison.

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RESULTS

Electrolyte studies

In contrast to desoxycorticosterone, triamcinolone exhibited a marked ability to promote sodium excretion when administered either subcutaneously or orally to adrenalectomizde rats (Tables 1 and 2). Rats receiving 50 μg. orally more than doubled their excretion of urine and increased uri-

TABLE 1. 5-HOUR URINARY EXCRETION FOLLOWING SUBCUTANEOUS ADMINISTRATION OF STEROIDS TO FASTED, SALINE-LOADED, ADRENALECTOMIZED RATS*

Compound	Dose, mg.	No.† rats	Urine volume		Electrolyte response			
					Na^+		K^+	
			ml./5 hrs. (S.E.)‡	% of cont.	m.eq./5 hrs. (S.E.)	% of cont.	m.eq./5 hrs. (S.E.)‡	% of cont.
Control	—	10	5.1 (±1.4)	100	1.55 (± .4)	100	0.20 (± .01)	100
DOC	.05	8	5.6 (± .37)	109	1.09 (± .12)	70	0.34 (± .02)	170
	.10	4	6.7 (6.0–7.5)§	131	1.25 (1.08–1.42)§	80	0.34 (.34–.34)§	170
Triamcinolone	.006	8	8.6 (±1.3)	168	1.74 (± .18)	112	0.30 (± .05)	150
	.025	8	15.4 (±1.3)	302	2.27 (± .18)	146	0.44 (± .03)	220
	.05	8	10.6 (±1.1)	208	1.93 (± .30)	124	0.55 (± .06)	275
	.10	8	11.7 (±0.77)	229	2.19 (± .15)	141	0.65 (± .03)	325

* Pooled data from duplicate experiments.
† 2 rats/cage—values expressed/cage.
‡ Standard Error.
§ Range.

TABLE 2. 5-HOUR URINARY EXCRETION FOLLOWING ORAL ADMINISTRATION OF STEROIDS TO FASTED, SALINE-LOADED, ADRENALECTOMIZED RATS*

Compound	Dose, mg.	No.† rats	Urine volume		Electrolyte response			
					Na^+		K^+	
			ml./5 hrs. (range)	% of cont.	m.eq./5 hrs. (range)	% of cont.	m.eq./5 hrs. (range)	% of cont.
Control	—	8	3.5 (2.5– 5.5)	100	0.96 (.75–1.26)	100	0.22 (.19–.26)	100
DOC	.05	4	4.0 (1.5– 6.5)	115	1.14 (.71–1.57)	118	0.30 (.29–.31)	137
	.25	4	3.0 (1.5– 4.5)	86	1.09 (.81–1.38)	113	0.23 (.22–.24)	105
	1	4	2.7 (2.5– 3.0)	80	0.89 (.87– .92)	92	0.21 (.20–.21)	95
	5	4	2.7 (2.5– 3.0)	80	0.82 (.74– .91)	85	0.43 (.37–.49)	195
Triamcinolone	.01	4	7.5 (6.0– 9.0)	215	1.36 (1.22–1.50)	142	0.21 (.20–.21)	95
	.05	8	7.9 (3.0–11.5)	226	1.55 (.88–2.09)	162	0.35 (.34–.39)	159
	.25	8	8.1 (7.0– 9.0)	231	1.57 (1.23–1.94)	163	0.51 (.46–.59)	232
	1.25	4	8.0 (5.0–11.0)	228	1.93 (1.84–2.02)	202	0.67 (.65–.69)	304

* Pooled data from duplicate experiments.
† 2 rats/cage—values expressed/cage.

nary sodium excretion by 62% as compared with adrenalectomized controls. At the same time, potassium loss was increased by 59%. Further evaluation of the data indicated that, generally, water and potassium excretion increased over control levels to a greater degree than that of sodium.

Comparable data on the diuretic and natriuretic actions of triamcinolone have been obtained by Dorfman *et al.* (28).

Liver glycogen deposition studies

Triamcinolone and prednisolone were compared with hydrocortisone for liver glycogen deposition activity after subcutaneous or oral administra-

tion (Tables 3 and 4). Triamcinolone was found to be 10 (95% fiducial limits 7 to 15) to 40 (23 to 69) times as potent as hydrocortisone subcutaneously, and 41 (30 to 56) to 84 (60 to 117) times hydrocortisone orally. The relative potency of triamcinolone to prednisolone in the subcutaneous and oral assay was calculated to be 3 (2 to 5) to 12 (8 to 20), and 9 (7 to 13) to 19 (14 to 27), respectively. Triamcinolone diacetate was found to be 9

TABLE 3. LIVER GLYCOGEN DEPOSITION FOLLOWING SUBCUTANEOUS ADMINISTRATION OF STEROIDS TO FASTED, ADRENALECTOMIZED RATS—7-HOUR ASSAY*

Compound	Dose (mg./rat)	No. of rats	Mean liver glycogen %	Standard error of mean
Control	—	14	0.25	± .02
Hydrocortisone	.25	4	0.52	± .07
	.50	4	1.04	± .04
	1.00	12	1.39	± .12
Prednisolone†	.125	8	0.83	± .10
	.25	6	1.55	± .27
	.50	8	1.54	± .01
	1.00	7	1.64	± .06
Triamcinolone	.006	8	0.36	± .08
	.025	7	0.79	± .09
	.050	8	1.15	± .06
	.100	8	1.32	± .07
Triamcinolone diacetate	.006	7	0.31	± .04
	.025	8	0.95	± .04
	.050	8	1.06	± .08
	.100	8	1.19	± .03

* Pooled data from duplicate experiments.
† Obtained from Schering Corp., Bloomfield, N. J.

TABLE 4. LIVER GLYCOGEN DEPOSITION FOLLOWING ORAL ADMINISTRATION OF STEROIDS TO FASTED, ADRENALECTOMIZED RATS—7-HOUR ASSAY*

Compound	Dose (mg./rat)	No. of rats	Mean liver glycogen %	Standard error of mean
Control	—	8	0.22	± .01
Hydrocortisone	2	4	0.99	± .23
	6	8	1.33	± .18
	12	8	1.55	± .10
Prednisolone†	0.5	8	0.79	± .03
	2	8	1.52	± .16
	8	8	2.19	± .18
Triamcinolone	0.01	4	0.43	± .06
	0.05	8	1.05	± .10
	0.25	8	1.35	± .14
	1.25	4	1.37	± .15
Triamcinolone diacetate	0.01	4	0.34	± .03
	0.05	8	0.82	± .08
	0.25	8	1.51	± .17
	1.25	4	1.34	± .23

* Pooled data from duplicate experiments.
† Obtained from Schering Corp., Bloomfield, N. J.

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(6 to 14) to 37 (22 to 63) times as potent as hydrocortisone subcutaneously, and 36 (26 to 49) to 73 (53 to 102) times hydrocortisone orally.

Life maintenance and growth studies

The data presented in Table 5 (Experiments 1 and 2) are the results of duplicate studies. Adrenalectomized rats had a mean survival time of 5 days. In groups receiving desoxycorticosterone, mean survival times of 7 to 16 days were recorded. Only the sham-operated animals survived the 28-day test. It is evident that hydrocortisone was less effective than desoxycorticosterone in maintaining life, while triamcinolone, triamcinolone diacetate, and prednisolone were at least as effective, if not more so. The results with prednisolone are in accord with those reported by other workers (6).

Changes in body weight observed during steroid treatment are included

TABLE 5. SURVIVAL TIME OF ADRENALECTOMIZED, IMMATURE MALE RATS

Treatment	Dose, mg./rat /day subcut.	No. of rats	Average body weight in gm.		No. surviving 14 days	Mean survival time (days) ±S.E.
			Initial (range)	At 13 days (range)		
			Experiment 1*			
Sham-adrenalectomized controls	—	15	60 (45–70)	130 (114–154)	13	25 ± 2.0
Adrenalectomized controls	—	15	60 (50–70)	—	0	5 ± .6
Desoxycorticosterone	0.045	15	59 (48–73)	—	0	7 ± .8
	0.18	16	56 (46–60)	104 (84–118)	11	16 ± 1.7
Hydrocortisone	0.12	16	52 (50–68)	96 (96)	1	10 ± .7
	0.48	15	60 (52–70)	93 (70–118)	12	17 ± 1.4
Prednisolone†	0.03	16	60 (47–72)	—	0	9 ± .7
	0.12	18	59 (50–71)	80 (58–100)	14	15 ± 1.2
Triamcinolone	0.015	15	59 51–64	—	0	8 ± .8
	0.03	15	58 (50–66)	70 (62–78)	3	9 ± 1.0
	0.06	17	58 (46–67)	69 (50–85)	12	13 ± 1.4

* Wistar strain rats.
† Obtained from Schering Corp., Bloomfield, N. J.

(*Continued on next page*)

TABLE 5. (*Continued*)

Treatment	Dose, mg./rat /day subcut.	No. of rats	Average body weight in gm.		No. surviving 14 days	Mean survival time (days) ±S.E.
			Initial (range)	At 13 days (range)		
	0.12	14	59 (52–69)	68 (60–74)	5	9 ± 1.7
Triamcinolone diacetate	0.015	16	57 (49–67)	56 (56–56)	1	8 ± 0.7
	0.03	16	58 (48–67)	65 (60–69)	3	8 ± 1.2
	0.06	17	58 (52–66)	68 (64–76)	7	11 ± 1.6
	0.12	15	58 (46–65)	64 (54–78)	7	12 ± 2.3
			Experiment 2*			
Sham-adrenalectomized controls	—	33	65 (52–78)	131 (103–170)	33	28
Adrenalectomized controls	—	35	62 (52–74)	94 —	1	5 ± .4
Desoxycorticosterone	0.045	35	65 (54–74)	93 (92–94)	2	7 ± .5
	0.09	33	62 (50–74)	91 (83–98)	8	9 ± .7
	0.18	34	62 (44–74)	102 (73–126)	21	14 ± 1.2
Hydrocortisone	0.12	34	62 (52–70)	88 (79–94)	4	9 ± .6
	0.24	34	64 (53–74)	88 (70–130)	26	15 ± .8
	0.48	34	63 (50–74)	94 (78–110)	25	15 ± 1.2
Prednisolone†	0.03	36	63 (54–70)	90 (74–105)	6	9 ± .8
	0.06	33	60 (55–72)	89 (74–112)	8	10 ± .8
	0.12	36	62 (50–73)	83 (56–108)	26	14 ± 1.0
Triamcinolone diacetate	0.015	34	63 (54–74)	79 (68–93)	6	9 ± .7
	0.03	32	59 (54–74)	78 (70–88)	13	12 ± .8
	0.06	34	63 (48–72)	70 (54–94)	22	14 ± 1.0
	0.12	32	63 (52–72)	70 (48–86)	24	17 ± 1.1

in Table 5. Desoxycorticosterone was superior to triamcinolone, triamcinolone diacetate, or prednisolone in promoting the growth of adrenalectomized rats. Moreover, prednisolone appeared superior to triamcinolone or its diacetate in this respect. That a direct relationship between survival and weight gain in adrenalectomized animals need not exist has been expressed by both Dorfman (29) and Leathem (30).

Anti-inflammatory studies

The ability of triamcinolone to inhibit the formation of granulomatous tissue appeared enhanced in comparison with hydrocortisone. It can be seen from the data (Table 6) that there was a decrease in granuloma formation as the dosage of steroid was increased. There was also a concomitant decrease in body weight.

TABLE 6. EFFECT OF STEROID TREATMENT ON GRANULOMA FORMATION

No. animals per group	Chemical	Dose, mg./day	Avg. body weight change, gm.	Avg. granuloma wt. per rat, mg.
12	Control	—	+10.8	1,202
6	Hydrocortisone acetate	12	−13.3	596
6		4	−11.6	682
6		1.4	− 6.6	832
6		0.4	− 3.3	1,064
6		0.14	+10.0	1,166
6	Triamcinolone	1.2	−28.3	552
6		0.4	−15.0	754
6		0.14	− 1.6	916
6		0.04	+ 3.3	1,152

While a decrease in both granuloma formation and body weight occurred during hydrocortisone and triamcinolone treatment, the two phenomena are not necessarily dependent upon each other. Desaulles *et al.* (31) have shown that, while cortisone and hydrocortisone result in a reduction of both granuloma formation and body weights, corticosterone and desoxycorticosterone reduced the granuloma formation while maintaining body weight gains.

The relative potency of triamcinolone was 5 (95 per cent fiducial limits 3 to 8) to 10 (7 to 15) times that of hydrocortisone.

DISCUSSION

Glucocorticoid potency exceeding that of cortisone and hydrocortisone has been demonstrated for 9α-halogenated steroids (1, 2), for compounds methylated at carbon 2 or those possessing both substituents (8), and by 9α-halogenated steroids containing a double bond between carbons 1 and 2 (11). However, each structural alteration alone or in combination greatly accentuated their ability to induce sodium retention.

The present report indicates that the 16α-hydroxylation of 9α-fluoro-Δ^1-hydrocortisone produced no major alteration of its glucocorticoid activity (11), yet completely negated its sodium-retention property. Triamcinolone not only failed to induce water and sodium retention, but actually acted as both a diuretic and natriuretic agent in the saline-loaded, adrenalectomized rat.

A further consideration of these data illustrates the potency of triamcinolone as a glucocorticoid. Subcutaneously administered triamcinolone revealed potencies relative to hydrocortisone and prednisolone of 10 to 40, and 3 to 12, respectively. However, orally, the relative potencies were 41 and 84, and 9 to 19, respectively. This enhanced oral-to-parenteral activity ratio also has been reported by Spero *et al.* (32) for the fluorinated 6-methyl steroids, and by Lyster *et al.* (10) for prednisolone and its 6-methyl analog. Though at present there are no experimental data to explain this phenomenon, these variances may reflect differences in the rates of absorption and/or metabolism of synthetic glucocorticoids.

Life maintenance and growth studies showed that hydrocortisone was less effective than DOC in maintaining the life of adrenalectomized rats, while triamcinolone and prednisolone were at least as effective. Both DOC and prednisolone were superior to triamcinolone in increasing the weight gain of these animals.

While it is difficult to make definite conclusions from these data concerning the relationships of the physiological activity of these steroids to the growth and longevity of adrenalectomized rats, some inferences can be drawn. Using triamcinolone as an example, it is quite apparent that liver-glycogen deposition and sodium excretion are not necessarily adverse to increased longevity in rats. Borman *et al.* (33) present evidence, using halogenated corticoids, that there is not always a direct correlation between sodium-retaining activity and growth-survival activity. 9α-bromo-hydrocortisone acetate, which has little sodium-retaining activity, markedly stimulated growth and survival, while 9α-fluoro-hydrocortisone acetate, which is more potent than DOC as a sodium retainer, and also possesses strong glucocorticoid activity (2), appeared less effective than DOC in growth-survival tests. To further complicate this picture, species differences between rats (33) and dogs (34) have been reported in life-maintenance studies with 9α-halogenated corticoids. Thus, it is apparent that, though salt conservation may be a predominant factor in life maintenance of adrenalectomized rats, protein-carbohydrate metabolism may play a role of considerable importance. This does not preclude the entrance of other, as yet unknown, factors.

A number of structural alterations in the steroid nucleus have been studied in relation to anti-inflammatory activity as measured by granuloma inhibition (35). The presence of various hydroxyl groups (11β, 17α, 21) appears essential for maximum anti-inflammatory potency. Fluorina-

tion of the 9α position of hydrocortisone further increased anti-inflammatory activity. In the cotton pellet anti-inflammatory assay Dulin (36) has reported that Δ^1-9α-fluoro-hydrocortisone is 14 times more active than hydrocortisone. Data obtained from the asbestos-pellet assay employed here indicate that triamcinolone is 5 to 10 times more active than hydrocortisone acetate. Since no direct comparison of triamcinolone and Δ^1-9α-fluorohydrocortisone was attempted, it is not possible at present to apply significance to the apparent depression in anti-inflammatory activity resulting from 16α-hydroxylation.

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EFFECTS OF RESTRICTED FOOD INTAKE AND GROWTH HORMONE ON RAT LIVER PROTEINS AND NUCLEIC ACIDS[1]

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ABSTRACT

A study was made to determine the part played by food intake on differences observed in rat liver cells of hypophysectomized and growth hormone treated hypophysectomized rats. It was found that: (1) neither restricted food intake nor growth hormone has any effect on the average DNA content of liver nuclei within a given class of ploidy; (2) restricted food intake does play some part in the degree of ploidy shift in the intact as well as in the growth hormone treated hypophysectomized animals. In order to obtain the full shift, both the presence of the pituitary (growth hormone) and an unrestricted food intake are necessary; (3) the inhibitory effect of restricted diet and the stimulatory effect of growth hormone on nuclear and cytoplasmic RNA and protein have been shown.

A differential effect of hypophysectomy on decreased nuclear RNA between diploid and tetraploid nuclei has been demonstrated, and a possible explanation for this effect has been offered.

An attempt has been made to relate these findings to the amino acid transfer hypothesis of growth hormone action.

THE results of a series of studies from this laboratory (1, 2, 3), on livers of young hypophysectomized rats, may be summarized as follows: (1) the amount of deoxyribose nucleic acid (DNA) per nucleus is unaltered by loss of the anterior pituitary hormones or by replacement therapy with a purified growth hormone preparation.[2] (2) The amount of nuclear and cytoplasmic protein and ribose nucleic acid (RNA), as well as nuclear and cytoplasmic volumes are lower than in intact controls of the same age and sex, while treatment of hypophysectomized animals with a purified growth hormone preparation results in a restoration to levels observed in intact controls. (3) There is an arrest of the "ploidy shift," which normally occurs during the growth of the animal, coupled with a marked reduction of mitotic activity. This results in a ploidy pattern like that found at the

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[2] The purified growth hormone (Lot No. M108) was generously supplied by the Endocrine Research Division of Armour and Company. Their analysis of this preparation indicated a complete lack of ACTH and gonadotrophic activities and the presence of a barely detectable TSH activity. The lack of gonadotrophic and ACTH activities was confirmed in our laboratory by the lack of increase in adrenal and testes weights in mg./100 gm. body wt. of hypophysectomized animals treated with the preparation as compared to operated controls treated with saline.

time of hypophysectomy. Growth hormone replacement therapy restores both ploidy distribution pattern and mitotic activity to those of intact controls of comparable weight and sex.

Low protein diets and starvation for varying periods of time have been reported to reduce cell basophilia and to cause other alterations in the chemical morphology of liver cells (4, 5, 6, 7, 8). Therefore, it was pertinent to determine whether or not differences in food intake between growth hormone treated and untreated hypophysectomized rats could be responsible in part for the changes previously reported by us.

MATERIALS AND METHODS

Hypophysectomized and intact male Sprague-Dawley rats were obtained one day after operation from the Hormone Assay Laboratory, Chicago, Illinois. The animals were observed for one day and then divided into 6 groups. The experimental design can be seen best by examination of the following table (Table 1):

TABLE 1. EXPERIMENTAL DESIGN

Group No.	No. of rats	Description	Time of tissue sampling
I	6	Initial intact	Mean body wt. as in II
II	5	Initial hypophysectomized	2 days post-op.
III	10	Intact, fed *ad lib.* for 7 days	8th day
IV	18	Hypophysectomized, fed *ad lib.* for 7 days,	8th day
V	7	Hypophysectomized, 0.25 mg. b.d. G.H. i.m., 7 days, pair-fed with IV	8th day
VI	13	Intact, pair fed with IV for 7 days	8th day

All animals were fed a measured amount of synthetic diet[3] adequate to support normal growth in intact rats of the age, sex and weight range used in these experiments (10 controls, average body weight 90 gm., fed the above diet for 7 days gained an average of 43 gm.).

The animals were sacrificed by decapitation; the livers were rapidly dissected out, blotted, weighed and samples for microspectrophotometric analysis were fixed in 25% neutral formalin. The remainder of the liver was then frozen in alcohol-dry ice and stored at −20° C for further biochemical analyses.

Cytochemical analyses were carried out on 10 μ paraffin sections. The cytophotometric apparatus employed for measurements of protein and nucleic acids *in situ* was a modification of one described by Pollister and Moses (9). The photometer used was a Photovolt Electronic Multiplier Photometer (Model 520M with a 21c search unit).

Determinations of DNA were made by measuring extinctions at 560 mμ of cores, 3 μ in diameter, through the centers of nuclei stained by means of the Feulgen reaction (14 min. hydrolysis in 1N HCl at 60° C, followed by staining with Schiff reagent prepared according to the directions of Stowell (10)). These extinction values were then converted to arbitrary units of DNA per nucleus according to the method described by Swift (11). Whole nuclei were selected at random for studies of ploidy distribution. For each group of experimental animals, the mean DNA content per diploid (2n) and tetraploid (4n)

[3] Casein 22%, Wesson oil 5%, Glucose 69%, Salts 4%. Vitamins/100 gm. of diet: Choline 50 mg., Paraaminobenzoic acid 2 mg., Inositol 50 mg., Thiamine 0.5 mg., Riboflavin 1.0 mg., Niacin 2.5 mg., Pyridoxine 0.5 mg., Calcium Pentothenate 5.0 mg., Biotin 0.4 mg., Menodione 0.5 mg., Folic acid 0.2 mg., Vit. B_{12} 12 μg., Vit. A 1,000 I.U., Vit. D 100 I.U., Alpha tocopherol acetate 10 mg.

class of nuclei was calculated from frequency distribution plots of all nuclei measured.

RNA studies were conducted utilizing basic staining in conjunction with ribonuclease digestion. Sections were digested for 3 hrs. at 37° C in a 0.1% aqueous solution of the enzyme (Armour and Co., Lot 9044X) which had been boiled to remove any traces of proteolytic activity and adjusted to pH 6.0 with NaOH. Control sections were similarly treated except for the absence of the enzyme from the incubating medium. Both control and digested sections were then stained with Azure A (National Aniline Certification No. NAz14) as described by Flax and Pollister (12) and Pollister (13). Thus nuclear and cytoplasmic RNA were computed by subtracting the amount of dye bound after ribonuclease digestion from that found in control sections.

For protein studies, the trichloracetic Millon reaction described by Pollister and Ris (14) was employed.

Extinction measurements of 3 μ plugs through nuclei and cytoplasm were made at 575 mμ for Azure A stained sections and 365 mμ for Millon stained sections. These measurements were made in cells whose nuclear size limits were calculated[4] to insure choosing cells which would be known to fall in each of the two ploidy classes studied (2n and 4n). Since the thickness of the sections used was greater than that of nuclei measured, nuclear extinctions were corrected for the extinction due to cytoplasm in the light path above and below the nucleus. These figures were then converted to arbitrary units of nuclear content as before (11). No attempt was made in these experiments to convert the extinctions measured for cytoplasmic plugs into units of content. These were utilized as extinction values, representing a measure of cytoplasmic concentration rather than content.

The desired wavelengths of light for measurement of extinctions of Azure A and Feulgen stained sections were isolated from a tungsten source by means of a 250 mm. Bausch and Lomb grating monochromator, while for Millon protein determinations the desired wavelength was isolated from a mercury vapor source.

Student's "t" test was used to test for differences between group means at the 1% level. "t" values were calculated from the total number of measurements on individual cells within each experimental group.

RESULTS

Body Weight: The average body weights of the 6 groups of animals at the time of sacrifice were as follows: *Group I*, initial intact, 96 gm.; *Group II*, initial hypophysectomized, 89 gm.; *Group III*, intact fed *ad lib.* for 7 days, 133 gm. (an average gain of 43 gm.); *Group IV*, hypophysectomized fed *ad lib.* for 7 days, 85 gm. (an average loss of 7 gm.); *Group V*, growth hormone treated hypophysectomized pair-fed against IV for 7 days, 110 gm. (an average gain of 14 gm.); *Group VI*, intact pair-fed against IV for 7 days, 90 gm. (an average gain of 6 gm.). The food intake of hypophysectomized animals, and therefore of animals pair-fed against them, was from 35 to 40% of that eaten by the *ad lib.* fed intact controls.

DNA and Ploidy Distribution: Table 2 summarizes the microspectrophotometric data of DNA content of 2n and 4n nuclei and the per cent distributions of these two classes. In all groups, 2n figures clustered around 9 DNA units and 4n figures around 18 DNA units. It is also apparent that, while there was no change in DNA content within a given class of ploidy,

[4] From ploidy distribution data by determining the ranges of nuclear diameters at which no overlap between 2n and 4n DNA values was observed.

TABLE 2. DNA CONTENT AND PLOIDY DISTRIBUTION OF RAT LIVER CELLS

Group No.	No. of cells	Content*		Per cent distribution*	
		2n	4n	2n	4n
I	400	9.31 ±0.08	18.2 ±0.2	56.3 ±1.5	43.7 ±1.5
II	400	10.1 ±0.1	19.3 ±0.2	62.5 ±1.4	37.5 ±1.4
III	400	9.81 ±0.08	19.2 ±0.2	37.5 ±1.2	64.3 ±1.2
IV	800	9.54 ±0.04	18.9 ±0.1	69.2 ±0.8	30.8 ±0.8
V	400	9.54 ±0.07	18.6 ±0.2	45.1 ±1.3	54.9 ±1.3
VI	400	9.13 ±0.09	18.0 ±0.2	42.6 ±1.1	57.4 ±1.1

* Each figure represents a mean value for the group ±S.E.

alterations in the per cent distributions of the two classes did occur. Diploid nuclei predominated in the ploidy distribution pattern in groups I, II, and IV, while tetraploid nuclei predominated in groups III, V, and VI.

RNA: The data on nuclear and cytoplasmic RNA measurements are presented in Table 3. A differential response of the RNA content in 2n as opposed to 4n nuclei was observed. This was not apparent in the cytoplasm of these two classes of cells. Attention is directed to the fact that in animals 2 days after hypophysectomy the nuclear content and cytoplasmic concentration was below that of initial intact rats (I *vs* II, $P < 0.01$); and that both were further decreased during the following 7 days (II *vs* IV, $P < 0.01$). The nuclear content and cytoplasmic concentration remained essentially unchanged in intact rats allowed food *ad lib.* for the 7 day experimental period (I *vs* III, $P > 0.1$). In pair-fed intact animals, during the same period of time, both nuclear content and cytoplasmic concentration of RNA were less than that of the *ad lib.* fed (III *vs* VI, $P < 0.01$). This decrease however, was not of the same magnitude as that seen in untreated hypopophysectomized animals (IV *vs* VI, $P < 0.01$).

Protein: The results of the protein studies are shown in Table 4. Differences in protein may be seen in both the nucleus and cytoplasm of the two ploidy classes of cells studied. Within each ploidy class the per cent change in all groups was the same for both nuclei and cytoplasm. Except for the lack of a differential response in protein between 2n and 4n nuclei which was observed in the RNA studies, the protein data paralleled the RNA data.

TABLE 3. NUCLEAR AND CYTOPLASMIC RNA OF RAT LIVER CELLS

Group No.	No. of cells	Nucleus				Cytoplasm			
		Content*		Per cent of initial intact		Extinction*		Per cent of initial intact	
		2n†	4n‡	2n	4n	2n†	4n‡	2n	4n
I	200	4.95 ± .09	7.92 ± .10	100	100	.426 ± .015	.451 ± .018	100	100
II	200	3.19 ± .06	6.05 ± .08	64	76	.255 ± .011	.257 ± .012	60	61
III	200	5.17 ± .08	8.25 ± .11	104	104	.439 ± .019	.473 ± .015	103	105
IV	400	2.64 ± .03	5.90 ± .04	53	75	.217 ± .008	.223 ± .007	51	50
V	200	4.42 ± .09	6.72 ± .09	89	85	.325 ± .012	.352 ± .013	76	74
VI	200	3.96 ± .08	6.55 ± .10	80	83	.335 ± .012	.368 ± .015	79	75

* Each figure represents a mean value for the group ±S.E.
† Measured only nuclei or cytoplasm of cells whose nuclear diameters ranged between 5.5 and 6.0 μ.
‡ Measured only nuclei or cytoplasm of cells whose nuclear diameters ranged between 7.8 and 8.3 μ.

TABLE 4. NUCLEAR AND CYTOPLASMIC PROTEIN OF RAT LIVER CELLS

Group No.	No. of cells	Nucleus				Cytoplasm			
		Content*		Per cent of initial intact		Extinction*		Per cent of initial intact	
		2n†	4n‡	2n	4n	2n†	4n‡	2n	4n
I	200	6.21 ± .11	13.3 ± .15	100	100	.640 ± .008	.615 ± .008	100	100
II	200	5.64 ± .10	11.9 ± .14	90	89	.572 ± .009	.553 ± .008	89	90
III	200	6.41 ± .09	14.0 ± .15	103	105	.667 ± .011	.648 ± .010	104	105
IV	400	4.43 ± .06	10.1 ± .10	71	76	.465 ± .005	.433 ± .004	73	70
V	200	5.73 ± .09	12.4 ± .12	92	93	.581 ± .009	.560 ± .008	91	91
VI	200	5.54 ± .09	12.1 ± .16	89	91	.585 ± .008	.569 ± .009	91	93

* Each figure represents a mean value for the group ± S.E.
† Measured only nuclei or cytoplasm of cells whose nuclear diameters ranged between 5.5 and 6.0 μ.
‡ Measured only nuclei or cytoplasm of cells whose nuclear diameters ranged between 7.8 and 8.3 μ.

DISCUSSION

Under conditions of these experiments neither growth hormone nor restricted food intake influenced the DNA content of rat liver cells within a given class of ploidy. This is in agreement with our previous findings (1, 2, 3) and with the findings of other workers who, by means of other techniques, have shown a lack of dietary effects on the amount of DNA within a liver cell nucleus (15).

In an earlier publication Di Stefano, *et al.* (3), in livers of rats of the same strain, weights and sex as those used in these experiments, reported a "ploidy shift" from a predominance of 2n nuclei to a predominance of 4n nuclei in intact animals fed *ad lib.* for 7 days; an inhibition of this "ploidy shift" in the hypophysectomized rat; and a restoration of ploidy patterns to control levels following growth hormone replacement therapy of hypophysectomized animals allowed to eat *ad libitum*. These observations have been confirmed and extended in the present study. Our earlier conclusion for this failure of development of the "ploidy shift" was that the lack of growth hormone was responsible for the maintenance of the more juvenile state of ploidy characteristic of the liver at the time of hypophysectomy. In this respect the synthetic activities of the cell necessary for the cell division, which must be a prerequisite for a change from a 2n to a 4n predominance, would be markedly reduced or inhibited by the lack of the anterior pituitary.

The data presented in this paper indicate that the "ploidy shift" was significantly retarded in the pair-fed intact rats (compare groups III and VI, Table 2, $P < 0.01$). In the hypophysectomized animals given growth hormone while pair-fed against *ad libitum* fed hypophysectomized animals, the resultant ploidy pattern was the same as that observed in the pair-fed intact rat. That the major portion of the ploidy shift is growth hormone dependent is indicated by a comparison of the observed growth hormone effect (groups IV, V, and VI, Table 2; V *vs* VI, $P > 0.1$; IV *vs* V and/or VI, $P < 0.01$) with the dietary restriction effect referred to above. The order of magnitude of the hormone effect is clearly greater than the inhibitory effect of diminished food intake.

Dietary effects resulting in losses of liver tissue components following

periods of fast or of feeding protein deficient diets have been reported by several investigators. Kosterlitz (5) has reported the loss of 27% of the liver cytoplasm in rats restricted to a protein-free diet for a period of 7 days; Brachet, *et al.* (6), who compared fasted and non-fasted control mice, noted a marked reduction in cytoplasmic protein and RNA in the livers of animals fasted for 4 days; Lagerstedt (7) studied changes in rat liver cells produced by the quantitative variations in the protein content of the diet, ranging from complete starvation for a period of 5 days through feeding of high protein diets to starved animals, and found that in fasted animals, as well as in animals on low protein diets, the degree of nuclear basophilia was markedly reduced and that of the cytoplasm eventually was completely lost. These parameters, he found, could be reversed by restoring protein to the animal's diet. Thomson, *et al.* (8) reported that in rat liver, a 48 hour fast caused a reduction of lipid phosphorus, protein nitrogen, and RNA by 20 to 30%. Our findings are in agreement with those of these workers since we observed a decrease of the same order of magnitude in these parameters in pair-fed animals whose food intake was reduced by some 60% of that of *ad lib.* fed intact animals. Although a reduction of nuclear and cytoplasmic RNA and protein was seen in the liver cells of the intact rats whose food intake was restricted by pair-feeding against hypophysectomized rats, this decrease was not as great as that seen in the hypophysectomized animals. This points to the fact that a major portion of the decreased RNA and protein in the liver cells of the hypophysectomized rat was due to the lack of the anterior pituitary and only partially to the lowered food consumption of these animals. That moiety of RNA and protein loss which was due to hypophysectomy was restored to pair-fed intact control levels by growth hormone administration, while both growth hormone treatment and unrestricted food intake would appear to be necessary in order to return these values to normal levels (1, 2).

The mechanism of the differential response to hypophysectomy of the 2n and 4n nuclear RNA mentioned above is not known. Since the age of the animals used in these experiments is such that in the intact rat there is a rapid shift from 2n to 4n predominance, the mitotic rate must be higher in the 2n class in order to allow this shift to take place; therefore, it may be that any deficiencies in the synthesis of RNA are brought into relief in this class.

It must be emphasized once again that a description of the effect of a hormone on the reproductive and protein synthetic apparatus of the cell does not necessarily imply that the biochemical locus of action of the hormone is intimately related to effects described. However, if an understanding of the effect of growth hormone on cells is to be achieved, an attempt to relate the observations described herein to other known actions of the hormone is in order.

Recently Noall, *et al.* (16) have made the intriguing suggestion that growth hormone may have as its primary effect that of concentrative

transfer of amino acids intracellularly. Certainly, the lowering of blood amino acid nitrogen that occurs following the administration of growth hormone to the intact animal can be seen within a few minutes (17). In contrast, nucleic acid and protein changes of the type described in this report have not been seen as late as 12 hours after initiation of growth hormone treatment in the hypophysectomized rat (18). Since a number of investigators have suggested that amino acids play a catalytic role in the synthesis of RNA and possibly DNA as well (19, 20, 21) the amino acid concentrative transfer hypothesis becomes progressively more appealing. For, if one postulates that the nucleic acid synthetic activities of cells (and, secondarily, their mitotic activity) are to some extent controlled or regulated by the size of the intracellular free amino acid pool, the RNA and DNA effects of growth hormone we have described could easily be secondary to an effect of growth hormone on some cell membrane amino acid transfer process. The fact that food restriction has an inhibitory effect on RNA synthesis, protein synthesis and mitotic activity may be explained partly on the basis of diminished intracellular amino acid pool size and partly on that of a diminished availability of calories for the energetic requirements of synthetic reactions.

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STUDIES ON THE EFFECT OF STORAGE ON THE PURITY OF SODIUM IODIDE-I^{131} [1]

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ABSTRACT

Solutions of NaI^{131} upon storage show the presence of several bands (usually six inclusive of NaI^{131}) when chromatographed one dimensionally in an ascending technique with various solvent mixtures and radioautographed. The percentage of radioactivity in the bands other than NaI^{131} increases rapidly upon storage at room temperature. Incubation of NaI^{131} solutions under nitrogen for 1½ hours with 40 mg./ml. of cysteine at pH 4 or pH 7 or 10 mg./ml. of NaI^{127} or KI^{127} at pH 7 converges the extraneous bands into the NaI^{131} band. Preliminary studies rule out the possibility that the bands other than NaI^{131} are due to oxides of iodine or due to iodides other than NaI^{131}. Studies on the uptake of the NaI^{131} or the major extraneous band by day old chick thyroids indicate that 20% of the injected radioactivity of the extraneous band is collected in the thyroid in contrast to the 60% uptake of NaI^{131}.

SODIUM iodide-I^{131} (NaI^{131}) is widely used for thyroid metabolism studies. Taurog, Potter and Chaikoff reported (1) that radioiodide obtained from Oak Ridge shows faint extraneous bands upon chromatography and that addition of thiouracil, thiosulfate or sulfite eliminates their presence. Sodium iodide-I^{131} which is used for clinical studies[2], also shows several bands when chromatographed one dimensionally in an ascending technique using several different solvent systems. The precentages of radioactivity in the bands other than NaI^{131} increases rapidly upon storage. The present study is concerned with the effect of storage on the purity of NaI^{131} and with the factors influencing the conversion of the various extraneous components into NaI^{131}. Preliminary studies are reported on the nature of the bands other than NaI^{131}.

METHODS

Chromatography and Radioautography

NaI^{131} (Abbott Radio-Pharmaceuticals) after storage at room temperature for four weeks was spotted in 0.6 μc. amounts on Whatman No. 1 filter paper strips and developed

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[2] Furnished by Abbott Radio-Pharmaceuticals, Oak Ridge, Tennessee or by E. R. Squibb & Sons, New Brunswick, New Jersey.

one dimensionally in an ascending technique using the following solvent mixtures: (A) n-Butanol saturated with 3N NH_3OH; (B) n-Butanol:acetic acid:water::78:5:17; (C) Wet collidine; (D) isoamyl alcohol saturated with 2N NH_4OH; (E) n-Butanol:dioxane:2N NH_4OH:50:12.5:37.5; (F) Methanol:water::70:30. After 20 hours, the strips were air dried and radioautographed. The location of the NaI^{131} band on the radioautograms was determined by running control strips spotted with a solution of 0.5 mg. NaI^{127} and developed under the same conditions. The NaI^{127} band was detected by dipping the strip in a solution of ammoniacal silver nitrate. The various bands obtained after chromatography and radioautography of NaI^{131} solution using solvent A were cut out, eluted with 50% ethanol and the radioactivity in each band was measured in a well counter. The percentages of the radioactivity in the bands from the origin upwards were 19, 21, 16, 32, 4 and 8% respectively. Since solvent A gave the best separation of the extraneous bands from NaI^{131}, this solvent system was used throughout the present study.

Effect of Storage of NaI^{131} on the Conversion of NaI^{131} into the Extraneous Bands

Three separate batches of NaI^{131} solutions which were 1, 4 and 7 weeks old were chromatographed using solvent A and radioautographed. The results are shown in Figure 2. In a separate experiment, solutions of NaI^{131} were stored from 1 to 4 weeks under sterile conditions and also in a frozen state.

Factors Affecting Conversion of the Extraneous Bands into NaI^{131}

Solutions of NaI^{131} (4 weeks old) containing 40 mg./ml. of thiouracil, cysteine, homocysteine, glutathione or ascorbate were incubated under nitrogen at 37° C for 1½ hours. At the end of the incubation time an aliquot was spotted on Whatman No. 1 paper strips and chromatographed with solvent A and radioautographed. The results are shown in Figure 3. Glutathione and cysteine converge the extraneous bands into NaI^{131} as shown in Figure 3. The effect of pH on the ability of glutathione or cysteine to converge the extraneous bands into NaI^{131} was studied. The results are presented in Table 1. The recovery of radioactivity of the extraneous bands converged into the NaI^{131} band following incubations with cysteine (pH 7) was determined. The effect of different levels of NaI^{127} and KI^{127} in converging the extraneous bands from NaI^{131} solutions into NaI^{131} was also studied.

TABLE 1. EFFECT OF pH ON THE CONVERSION OF EXTRANEOUS BANDS INTO NaI^{131} BY CYSTEINE AND GLUTATHIONE

Substances incubated*	pH of the solutions	Distribution of radioactivity % in bands					
		1	2	3	4	5	6
NaI^{131} control	4	24	16	11	47	1	1
	7	24	17	11	45	2	1
	10	24	17	13	44	1	1
NaI^{131}+cysteine	4	1	0	97	0	1	1
	7	1	0	98	0	1	0
	10	9	1	72	18	0	0
NaI^{131}+glutathione	4	1	0	99	0	0	0
	7	5	1	85	7	1	1
	10	11	2	61	24	1	1

* Solutions of NaI^{131} (44 days old) containing 40 mg./ml. cysteine or the same amount of reduced glutathione were adjusted to pH 4, 7 or 10 and incubated under nitrogen at 37° C for 1½ hours. Aliquots containing 0.6 μc. of incubated material were chromatographed using solvent A, and radioautographed. The different bands were cut out, eluted and counted.

Studies on the Identity of the Extraneous Bands

Chromatograms were run using solvent A on NaI^{127} solutions or solutions of mixtures of NaI^{127} with ascorbate or bisulfite which were stored for 4 weeks. NaI^{127} band was identified by dipping the chromatograms in ammoniacal silver nitrate. The Rf. values of KI^{127}, ZNI_2^{127}, RbI_2^{127} and MgI_2^{127} with solvent A were found to be the same as that of NaI^{127}. The Rf. values of sodium iodate, sodium meta-periodate and sodium periodate with solvent A were determined. Effect of autoclaving on NaI^{127} or of x-irradiation (2,000γ) on NaI^{127} solution with regard to the presence or absence of extraneous bands was studied.

RESULTS AND DISCUSSION

The radioautographs of chromatograms of NaI^{131} solutions (4 weeks old) developed with the various solvent mixtures clearly indicate that upon storage, NaI^{131} solutions contain more than one band (Fig. 1). Solvents F and B do not resolve the different bands effectively but when developed with solvents A, C, D and E, NaI^{131} solutions separate into 5 or 6 bands. Of these, the NaI^{131} band is number 3 solvent A, number 2 with solvent B, number 4 with solvent C, number 2 with solvent D, number 4 with solvent E and number 3 with solvent F. The rest of the bands are extraneous components, probably formed from NaI^{131} on storage. The percentages of the total radioactivity in the six bands obtained by chromatographing NaI^{131} solutions with solvent A were 19% in band 1, 21% in band 2, 16% in band 3 (NaI^{131} band), 32% in band 4, 4% in band 5 and 8% in band 6 (Fig. 1, solvent A). Thus only 16% of the total radioactivity is in the NaI^{131} band while the rest is distributed among five other components.

The results shown in Figure 2, in which NaI^{131} solutions were stored at room temperature for different periods indicate clearly an increase in the radioactivity of the extraneous bands on storage. This was also true of NaI^{131} solutions stored under sterile conditions or in a frozen state. Since Na^{131} solutions are used extensively both clinically and for research purposes, it is extremely important to determine the identities and properties of the various extraneous substances formed in solutions of NaI^{131}. The NaI^{131} solutions which are freshly received or which are only a week old also contain a significant amount of radioactivity in band 4 as shown in Figure 2.

Of the several reducing agents tested (thiouracil, cysteine, homocysteine, glutathione and ascorbate) for their ability to converge the extraneous bands into NaI^{131}, cysteine and glutathione gave the best conversion as shown in Figure 3. That this is true "conversion" and not merely a disappearance of the extraneous substances was shown as follows: The radioactive substances making up the various bands both in the control strip and in the strip in which cysteine had converged the bands were eluted and counted. The radioactivity in the NaI^{131} band from the cysteine strip was 90% of the total counts of the individually eluted bands from the

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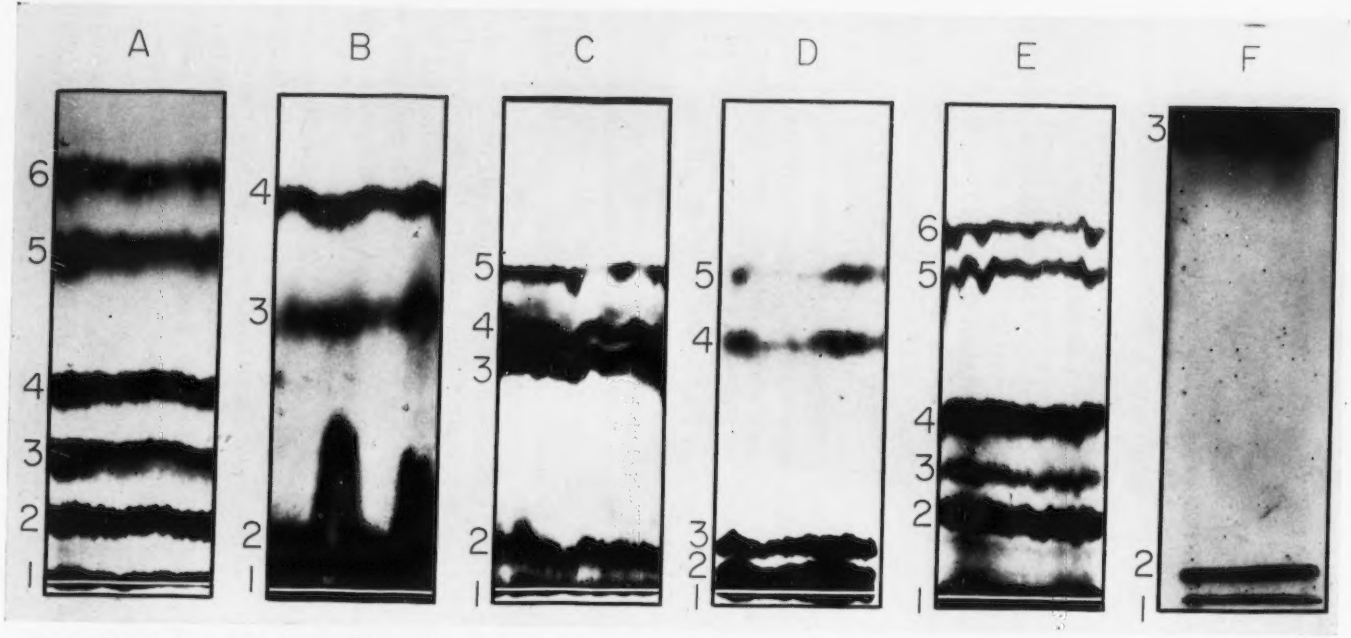

FIG. 1. Radioautographs obtained from chromatograms of NaI^{131} solutions (4 weeks old) developed with the following solvent mixtures: (A) n-Butanol saturated with 3N NH_4OH; (B) n-Butanol:acetic acid:water::78:5:17; (C) Wet collidine; (D) Isoamyl alcohol saturated with 2N NH_4OH; (E) n-Butanol:dioxane::4:1 saturated with 2N NH_4OH; (F) Methanol:water::70:30. The NaI^{131} bands are nos. 3, 2, 4, 2, 4, and 3 from the origin with solvents A through F respectively. The percentages of the total radioactivity in the different bands obtained with solvent A are as follows: bands 1 through 6 are 19, 21, 16, 32. 4 and 8% respectively.

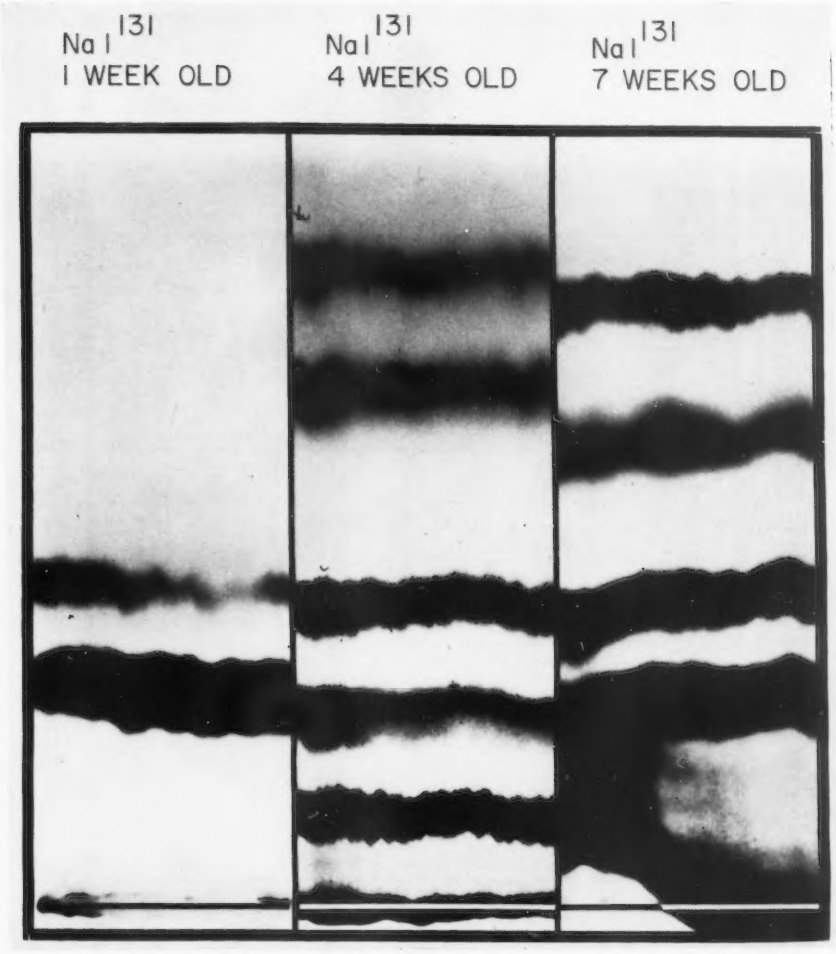

FIG. 2. Effect of storage of NaI^{131} solutions on the conversion of NaI^{131} into extraneous bands. Strips 1, 2 and 3 represent radioautographs of chromatograms of NaI^{131} solutions from batches which are 1, 4 and 7 weeks old respectively. The chromatograms were developed using solvent A.

control strip. The results of the study on the effect of pH on the ability of cysteine and glutathione to converge the extraneous bands into NaI^{131} are presented in Table 1. Eleven to 13% of the total radioactivity of NaI^{131} solutions is present in the NaI^{131} band, while the remaining 87% is distributed among 5 extraneous bands. Changes of pH's do not influence the percentage of the radioactivity in the NaI^{131} band in the control experiment. At pH 4, both glutathione and cysteine are effective in converging the extraneous bands into NaI^{131}. However, at pH 7, cysteine is superior to

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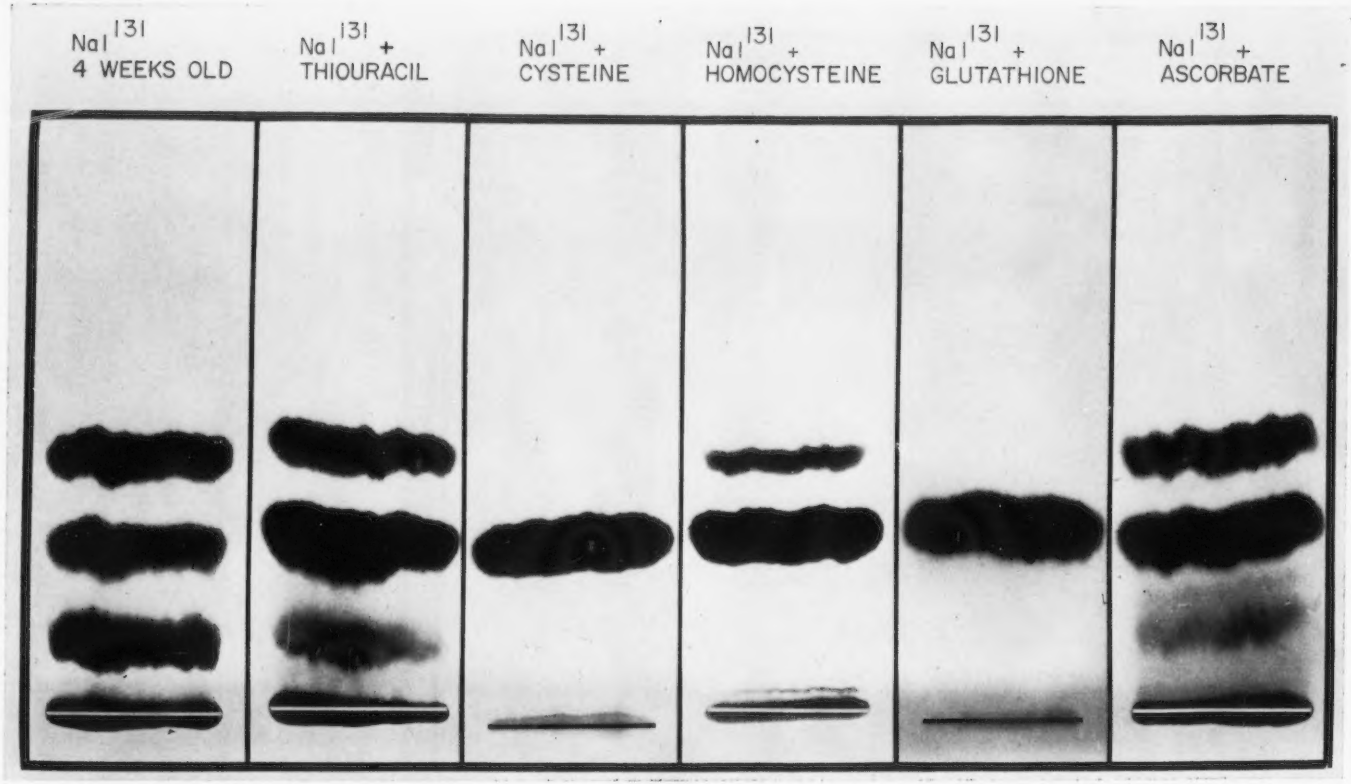

FIG. 3. The effect of reducing agents on the conversion of extraneous bands into NaI^{131}. Solutions of NaI^{131} (4 weeks old) containing 40 mg./ml. of thiouracil, cysteine, homocysteine, glutathione and ascorbate were incubated under nitrogen at 37° C for 1½ hours. An aliquot containing 0.6 μc. of the incubated material was chromatographed with solvent A and radioautographed.

glutathione. On the other hand, at pH 10, both cysteine and glutathione are less effective than at pH 4 or at pH 7.0. Incubations of NaI^{131} solutions with 10 mg./ml. of NaI^{127} or KI^{127} at pH 7.0 for $1\frac{1}{2}$ hours also effectively converges the extraneous bands into NaI^{131} band.

Aqueous solutions of NaI^{127} (1%) which have been stored at room temperature for 4 weeks still showed only one NaI^{127} band when chromatographed with solvent A. Addition of ascorbate or bisulfite to the NaI^{127} (1%) solutions do not affect the NaI^{127} band. Iodides with different cations (K, Zn, Rb and Mg) have the same Rf. value as the NaI^{127} band with solvent A. None of the extraneous bands observed in NaI^{131} solutions have the same Rf. values with solvent A as those of sodium iodate, sodium metaperiodate or sodium periodate. Solutions of NaI^{127} (1%) which are autoclaved for 15 minutes at 120° C give only one NaI^{127} band when chromatographed with solvent A. X-irradiation (2,000γ) of a solution of NaI^{131} does not increase the number or quantity of the extraneous bands. These studies rule out the possibility that the extraneous bands found in NaI^{131} solutions are iodides with different cations or that they are oxides of iodine.

Studies were conducted using day old chicks (New Hampshire males $\times$Single Comb White Leghorn females) to determine the uptake of NaI^{131} or the major extraneous band (band 4, Fig. 1, Strip A) by the thyroid. Several microcuries of NaI^{131} solutions (4 weeks old) were spotted on sheets of Whatman No. 1 filter paper and developed using solvent A and radioautographed. The extraneous band (No. 4, Fig. 1, Strip A) and NaI^{131} band were eluted from the chromatograms and evaporated under nitrogen to 10 μc./ml. concentration. Six chicks were injected in the breast muscle with 1 μc. each of the NaI^{131} and three were given 1 μc. each of the extraneous band. At the end of 24 hours, the animals were killed by decapitation, the thyroids were removed and the collection of radioactivity by the thyroids was determined using a well counter. The results indicate that the three chicks which received the extraneous band collected only 20% of the injected radioactivity, while the six chicks injected NaI^{131} collected on the average 60% of the injected dose.

Reports of further studies of the biological properties of each band will appear in a separate communication.

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NOTES AND COMMENTS

THYROXINE EXCRETION INCREASE BY COLD EXPOSURE[1]

ABSTRACT

Rats were maintained in a cold room at 10° C for 11 days. After injection of potassium perchlorate and I^{131} thyroxine, excretory depletion rates of radiothyroxine were measured for a 4 day period. During the first twenty-four hours, cold-exposed rats excreted twice as much fecal I^{131} as rats at room temperature. This suggests that the goitrogenic effect of cold may be related to thyroxine depletion by an increased rate of fecal excretion.

Fecal iodine is predominantly organic iodine (1) derived from the thyroid gland (2). Fecal excretion of I^{131} thyroxine has been reported to be greater in rats fed a high concentration of dietary bulk as compared to rats fed diets relatively low in bulk (3). This has been related to an interference with reabsorption of biliary thyroxine (3, 4). Reichlin (5) reported that starvation in rabbits decreased fecal I^{131} thyroxine excretion which accompanied decreased fecal volume. Since cold-exposure increases thyroxine requirement (6), thyroid size (7), and food consumption (8), it was reasoned that the increased fecal volume associated with the increase in food consumption may alter the loss of fecal thyroxine in cold exposed animals.

METHODS

A group of albino rats was exposed to $10 \pm 0.5°$ C for 11 days, another group was kept at $22 \pm 1°$ C as controls. Purina dog chow (iodine content 1.4 μg./gm.) was fed *ad libitum*. The control rats ate approximately 15 gm. of food per day and those at 10° C ate 25 gm. per day. This resulted in an iodine intake which was at least 10 times greater than the requirement. Both groups were injected intraperitoneally with 5 mg. potassium perchlorate solution. Thirty minutes later they were similarly injected with I^{131} L-thyroxine[2] (1.1 μg., 2.3 μc. in .05 cc. of propylene glycol). Potassium perchlorate (5 mg./day) was administered continuously in the diet for 4 days. The animals were kept in wire-bottom metabolism cages and daily excretion collected separately for 4 days after thyroxine injection. Thyroids were blocked with potassium perchlorate in preference to other antithyroid agents because Van Arsdel has suggested (9) that propylthiouracil increased fecal excretion of radiothyroxine but perchlorate did not alter the metabolism of the hormone. After radiothyroxine and perchlorate injections, synthesis of endogenous thyroxine was minimized and the body content of thyroxine began to be depleted. Iodine reserves were measured (11) in plasma and thyroid of similar rats not administered potassium perchlorate and thyroxine.

Fecal I^{131} has been shown to be predominantly thyroxine after radiothyroxine injection (1), therefore the rate of fecal excretion of I^{131} measured the excretory depletion rates of thyroxine from animals.

RESULTS

Table 1 shows the iodine reserves were similar in rats exposed to room temperature and the mildly cold environment of 10° C. Table 2 shows that during the first 24 hours, rats at 10° C excreted about twice as much fecal thyroxine as controls. After one day, the cold-exposed rats retained only 13% of the administered dose compared to 43% retention in the controls. Initial urinary radioiodide appeared to be greater in rats at 10° C but the large standard deviations prevented these urinary data from showing statistically significant differences. Fecal excretion of I^{131} thyroxine was not significantly different at the two temperatures during the second day. The fecal and urinary I^{131} excreted during the

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[2] Prepared by Abbott Laboratories.

TABLE 1. IODINE RESERVES OF CONTROL RATS EXPOSED TO 10° C AND 22° C FOR 37 DAYS (Diet, Purina Dog Chow)

No. of rats	Temp.	Protein-bound iodine	Thyroid iodine	Thyroid wt.
	° C	μg.%	μg.	mg./100 gm. body wt.
8	10°	4.7	13.1	7.9
8	22°	5.6	14.4	7.5

third day after injection was significantly greater in the control group. This reversal of radioiodine excretion is probably due to the relatively large initial retention in the control group. Since the cold-exposed rats lost the greatest percentage of the dose the first day, less radiothyroxine was available for subsequent excretion in the cold as compared to the amount available for excretion in the controls. Accumulative fecal radiothyroxine during the 4-day experimental period was 59.7% at 10° C and 45.9% at 22° C ($p < .02$). The data indicate that after 96 hours both groups have lost only slightly different amounts of organic iodine in the feces, but the animals exposed to 10° C lost it more rapidly. The effectiveness of thyroid blockade by perchlorate was shown by hypertrophy of the glands within 96 hrs. Among these animals the cold-exposed rats developed larger thyroids (18.7 mg./100 gm. body wt.) than the rats at 22° C (11.7 mg./100 mg. body wt.).

TABLE 2. EXCRETION OF I^{131} AFTER INTRAPERITONEAL INJECTION OF I^{131} THYROXINE; RATS EXPOSED TO 10° C AND 22° C

(Thyroids blocked with $KClO_4$)

No. of rats	Avg. body weight	Temp.	Excreta collection periods (hours after thyroxine injection)						Plasma I^{131} after 96 hrs.	Carcass I^{131} after 96 hrs.
			0–24		24–48		48–96			
			% of thyroxine dose							
	grams	° C	feces	urine	feces	urine	feces	urine	% dose/cc.	% dose
8	198	10°	47	40	10.6	7.7	2.1	1.5	.01	0.7
Standard Deviation			±17.5	±12.5	± 7.4	± 3.7	±1.4	± .8	± .005	± .38
7	200	22°	24.5	32	13.9	14	7.5	7.4	.05	3.1
Standard Deviation			±3.8	± 5.1	± 2.1	± 1.1	± .48	±1.1	± .018	± .60
p*			< .02	.2	.8	<.005	<.001	<.001	<.001	<.001

* p values from Fisher's t-test (10) applied to differences between means at 10° C and 22° C.

DISCUSSION

Rats exposed to 10° C for 11 days and then injected with radiothyroxine excreted I^{131} in the feces at a faster rate than controls at 22° C. This mild cold exposure was not goitrogenic in rats maintained on a high iodine intake. Thus, the increased thyroxine excretion occurring at 10° C can be adequately replenished by a high iodine diet. Animals on a lower iodine intake may not be able to replace this rapid thyroxine loss. Increased fecal thyroxine excretion in the cold should increase thyroxine requirements and thereby may be a major cause of the increased incidence of goiter in animals exposed to moderate cold. We consider that any factor which increases dietary intake and fecal volume might increase fecal thyroxine excretion.

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THE ANTI-ANDROGENIC ACTIVITY OF A PHENANTHRENE DERIVATIVE IN THE CHICK*

ABSTRACT

2-Acetyl-7-oxo-1,2,3,4,4a,4b,5,6,7,9,10,10a-dodecahydrophenanthrene when inuncted directly on the chick's comb inhibits the stimulating effect of injected testosterone.

Anti-androgenic agents may be evaluated by direct inunction on the chick's comb stimulated by testosterone incorporated into the diet (1). This communication reports the anti-androgenic activity of a nonsteroidal compound, 2-acetyl-7-oxo-1,2,3,4,4a,4b, 5,6,7,9,10,10a-dodecahydrophenanthrene (Ro-2-7239) by a modification of this assay procedure and confirms the anti-androgenic activity reported for this compound in the castrated rat by Randall and Selitto (2).

METHOD

Two-day old White Leghorn cockerels were injected subcutaneously with 0.5 mg. of testosterone enanthate contained in 0.5 ml. of sesame oil. On the same day and for the following six consecutive days the test compound dissolved in 0.05 ml. of sesame oil was inuncted on the comb once daily. One day after the last inunction the chicks were sacrificed and body weights and comb weights were determined. The results are expressed as comb ratios, defined as mg. of comb per gram of body weight.

RESULTS AND CONCLUSIONS

Norethisterone (1) (19-Nor-17-ethynyltestosterone) in amounts of 0.5 to 1.0 mg. significantly inhibited the stimulating effect of injected testosterone (Table 1). Table 2 illustrates the fact that Ro-2-7239 was approximately as active as norethisterone in inhibiting the action of testosterone. The mean comb ratios of groups of untreated chicks varied between 0.33 ± 0.011 and 0.38 ± 0.017. Thus the maximum inhibitory effects of norethisterone and Ro-2-7239 were 60% and 44% respectively. Ro-2-7239 does not ap-

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TABLE 1. THE ANTI-ANDROGENIC ACTIVITY OF NORETHISTERONE

(All chicks received a stimulating dose of 0.5 mg. of testosterone enanthate)

Test No.	Material inuncted	Total dose, mg.	No. of chicks	Mean comb ratio ±S.E.
1	0	0	44	0.93 ± 0.029
	Norethisterone	0.25	37	0.77 ± 0.028
		0.50	35	0.73 ± 0.025
		1.00	35	0.71 ± 0.030
2	0	0	25	1.02 ± 0.055
	Norethisterone	0.25	20	0.76 ± 0.035
		0.5	20	0.77 ± 0.048
		1.0	20	0.64 ± 0.025
3	0	0	29	1.23 ± 0.072
	Norethisterone	0.25	30	1.18 ± 0.066
		0.5	30	0.80 ± 0.042
		1.0	30	0.74 ± 0.031

TABLE 2. THE ANTI-ANDROGENIC ACTIVITY OF Ro-2-7239 COMPARED TO NORETHISTERONE

Test No.	Material inuncted	Total dose, mg.	No. of chicks	Mean comb ratio ±S.E.
4	0	0	24	1.08 ± 0.068
	Norethisterone	0.25	22	0.93 ± 0.041
		0.5	21	0.92 + 0.043
		1.0	23	0.85 ± 0.041
	Ro-2-7239	0.1	17	0.97 + 0.034
		0.2	17	0.92 + 0.042
		0.4	17	0.81 + 0.042
		1.6	13	0.73 ± 0.055
		5.0	12	0.74 ± 0.036
5	0	0	28	0.81 ± 0.037
	Norethisterone	0.25	23	0.67 ± 0.027
		0.50	23	0.65 ± 0.031
		1.00	24	0.67 ± 0.027
	Ro-2-7239	0.1	19	0.66 ± 0.069
		0.4	20	0.63 ± 0.025
		0.8	20	0.61 ± 0.044
6	0	0	19	1.02 ± 0.035
	Norethisterone	0.25	18	0.92 ± 0.049
		1.00	18	0.70 ± 0.049
	Ro-2-7239	0.25	20	0.69 ± 0.050
		1.00	20	0.74 ± 0.031

pear to have any other hormonal effects (2) and in this sense is comparable to other non-steroidal anti-androgens including methylcholanthrene (3) and 20-methyl-Δ^5-pregnene-3β,20-diol (4).

Acknowledgments

Thanks are due to Hoffmann-LaRoche, Inc., for the supply of Ro-2-7239, to Syntex S. A., Mexico City, for norethisterone, and to Mr. Albert Tomolonis and Miss Theresa Rutkiewicz for their valuable technical assistance.

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INHIBITION OF THYROXINE ACTION BY 3,3′,5′-TRIIODOTHYRONINE[1]

ABSTRACT

L-Thyroxine injected in doses of 10–20 μgm./kg./d. for 2–3 weeks into thyroidectomized rats maintained the BMR at about 20% above the hypometabolic level. DL-3,3′,5′-Triiodothyronine completely reversed the effect when used at 2 mg./kg./d. The increased oxygen consumption of tissues removed from thyroxine-injected hypothyroid animals could also be depressed by the concomitant injection of 3,3′,5′-triiodothyronine.

3,3′,5′-Triiodothyronine (3,3′,5′-Trit) has been encountered in the thyroid gland (1) and blood (2) of I^{131}-injected experimental animals. In contrast to its 3,5,3′-isomer, this compound has been found to produce less than 0.5% of the metabolism-stimulating effect of thyroxine (3). Since 3,5,3′-Trit is some four times as active as thyroxine, the 3,3′,5′-Trit can be considered inactive. Therefore it was thought of interest to evaluate its action in inhibiting the metabolic effect of thyroxine, particularly in view of the earlier report by Cortell (4) that 2′,6′-diiodothyronine possessed thyroxine-inhibiting properties.

METHODS

Thyroidectomized Sprague-Dawley rats were used, at least two months after operation. For studies on the BMR of the whole animal, post-absorptive rats on a special feeding regime (5) were trained to the chamber of an apparatus similar to that described by Watts and Gourley (6). After a series of satisfactory control determinations, selected animals were started on L-thyroxine[2] injected daily subcutaneously at 10 or 20 μg./kg. When the elevated BMR values became stabilized, usually after 3 weeks, the animals were given 3,3′,5′-Trit[3] subcutaneously in addition to thyroxine. The level of depressed metabolism was established for several days and the 3,3′,5′-Trit withdrawn, leaving the animals on thyroxine alone.

The tissue slice studies employed conventional Warburg oxygen consumption measurements on representative organs (liver, kidney, salivary glands, diaphragm, and heart) excised from thyroidectomized rats which had been injected for four days with one of the following: blank, thyroxine (0.25 mg./kg./d.), or thyroxine plus varying amounts of 3,3′,5′-Trit, (10 or 20 mg./kg./d.).

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[1] This investigation was supported by research grant A-1545 from the Institute for Arthritis and Metabolic Diseases, U. S. Public Health Service.

[2] L-Thyroxine was furnished by Dr. H. L. Fevold of the Travenol Division, Baxter Laboratories.

[3] 3,3′,5′-Triiodothyronine was provided by Drs. R. I. Meltzer and R. L. Kroc of the Warner-Chilcott Laboratories.

RESULTS

Figure 1 charts the time course of BMR response of a group of 3 thyroidectomized rats. Each point represents the mean oxygen consumption of the 3 animals under the treatment shown. The horizontal lines show the average value over the time limit indicated. For instance, line #1 indicates the control BMR at 88.9 ml. O_2/100 gm. body wt./hr. over the span of 7 days; line #2 at 108.8 ml. O_2, representing the plateau of response to 10 μgm. of thyroxine. When 2 mg. of 3,3′,5′-Trit was superimposed, the metabolic rate decreased to line #3, 86.2 ml. O_2 after 4 days. Upon cessation of 3,3′,5′-Trit, the BMR returned in the next 20 days to line #4, 108.5 ml. O_2. A second course of 3,3′,5′-

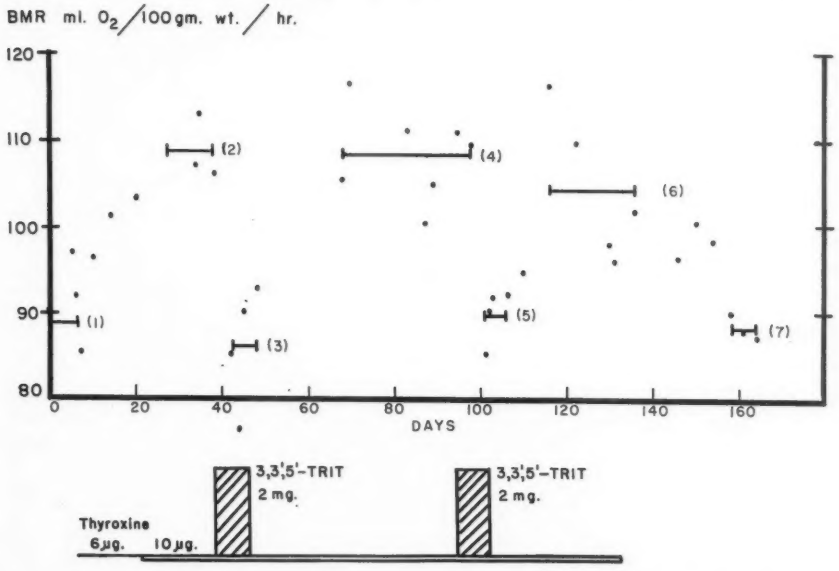

FIG. 1. Response of oxygen consumption of thyroidectomized rats to thyroxine and 3,3′,5′-triiodothyronine. The horizontal lines represent averages over the time periods indicated. The doses shown for thyroxine and 3,3′,5′-Trit are mg./kg./d.

Trit gave similar results. After withdrawal of the daily thyroxine, the BMR returned to its starting level after 2 weeks.

Results from this group of animals indicate a 100% inhibition of thyroxine maintenance of metabolism at a molar ratio of 200:1 for DL-3,3′,5′-Trit:L-thyroxine. Another group of 2 animals studied through one course of 3,3′,5′-Trit responded with a 93% inhibition at a molar ratio of 100:1.

The effects of 3,3′,5′-Trit on the tissue metabolism from rats injected with a larger dose of thyroxine are shown in Figure 2. Each value represents the average of 5 tissues, liver, kidney, salivary glands, diaphragm, and heart from 2 or 3 animals. Using blank-injected rats as controls, injections of 0.25 mg. of thyroxine per kg. for 4 days gave a 22.9% increase in tissue oxygen consumption. When 10 mg./kg. of 3,3′,5′-Trit was injected simultaneously along with the thyroxine, the increase of tissue oxygen consumption was 18.7%, while the increase was only 7.4% in rats receiving 20 mg. 3,3′,5′-Trit per kg. in a similar fashion.

The results from tissue studies indicate a 9.6% inhibition of thyroxine effect at a

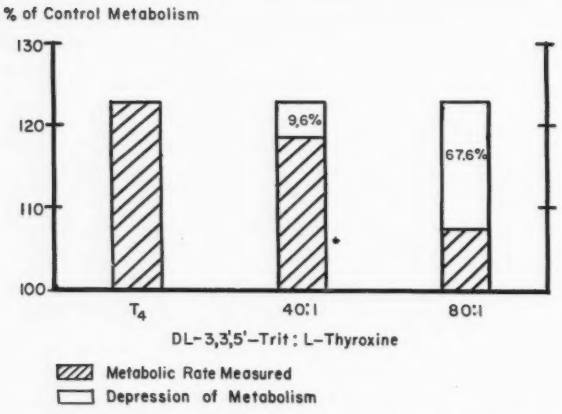

FIG. 2. Summated tissue oxygen consumption of hypothyroid rats treated with thyroxine alone or combined with 3,3′,5′-triiodothyronine. Thyroxine dosage 0.25 mg. /kg./d., 3,3′,5′-Trit at molar ratios of 40 or 80.

molar ratio of 40:1 for DL-3,3′,5′-Trit: L-thyroxine and a 67.6% inhibition at a molar ratio of 80:1.

DISCUSSION

It is apparent that DL-3,3′,5′-Trit in an amount 80 to 100 times that of L-thyroxine is able to interfere with the metabolic effects of the hormone. In all experiments the inhibitory effect was titrated against exogenous thyroxine in completely thyroidectomized animals. Consequently, the applicability of our findings to the therapy of euthyroid or hyperthyroid states seems highly questionable. Preliminary evaluation in athyreotic patients maintained on desiccated thyroid, thyroxine or Trit shows a considerable depression of BMR, but no effect in euthyroid subjects (personal communication from Dr. S. R. Hill).

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ANNOUNCEMENTS

ENDOCRINOLOGY STUDY SECTION

The Endocrinology Study Section, National Institutes of Health, announces that it is prepared to distribute, free of charge to qualified investigators, limited amounts of purified sheep pituitary follicle-stimulating hormone. The preparation has been packaged in two forms. 1) vials containing 5 mg. for use only as a Standard in assay procedures; 2) vials containing 25 mg., for experimental use. The preparation is a sterile, lyophilized powder, free or nearly free of other hormonal activities. Detailed specifications will be distributed with each sample.

Application for supplies, either of the Standard or of the experimental lots, should be made by letter to Dr. R. T. Hill, Executive Secretary, Endocrinology Study Section, National Institutes of Health, Bethesda 14, Maryland. The letter should state briefly an outline of the intended work and an estimate of the amount needed. Applications will be screened by a subcommittee and allotments will be made according to the estimates of merit of the intended work.

The preparation and distribution of FSH is supported by funds granted by several Institutes of the National Institutes of Health. The program is supervised by a subcommittee consisting of Drs. Warren O. Nelson, Roy W. Hertz, Robert W. Bates, R. T. Hill, and Alfred E. Wilhelmi, Chairman. The hormone preparation was made at Emory University under the immediate direction of Dr. Stanley Ellis.

1959 LAURENTIAN HORMONE CONFERENCE

The 1959 Laurentian Hormone Conference of the AAAS will be held at Mont Tremblant Lodge, Mont Tremblant, Quebec, during the period August 30–September 4. Investigators interested in attending this Conference should make application to the Committee on Arrangements of the Laurentian Hormone Conference, 222 Maple Avenue, Shrewsbury, Massachusetts, at an early date and in any event no later than May 11, 1959. A conference rate of $13.00 per day per person is extended to all invited participants. Since the number of our participants is necessarily limited by available accommodations, all applications are screened and invitations to attend are issued by the Committee on Arrangements by June 12. The following program has been arranged.

LAURENTIAN HORMONE CONFERENCE, INC.

Mont Tremblant Lodge

AUGUST 30–SEPT. 4, 1959

I. MECHANISM OF HORMONE ACTION

Participation of steroid hormones in the enzymatic transfer of hydrogen.
Paul Talalay and H. G. Williams-Ashman, The Ben May Laboratory, University of Chicago

Sunday evening, August 30

An enzymatic basis for the physiologic function of estrogens.
Claude A. Villee and Dwain D. Hagerman, Harvard University Medical School, Boston

Sunday evening, August 30

Effects of steroid hormones on some coenzyme mediated enzymatic reactions.
Lewis L. Engel and Jesse F. Scott, John Collins Warren Laboratories of the Huntington Memorial Hospital of Harvard University, Massachusetts General Hospital, and Departments of Biological Chemistry and Anatomy, Harvard Medical School

Monday morning, August 31

The mechanism of action of some estrogenic hormones on metabolism.
Kenneth W. McKerns, Edwin Kaleita and Paul H. Bell, Lederle Laboratories, American Cyanamid Company, Pearl River, New York

Monday morning, August 31

The role of cyclic adenylic acid in hormone action.
Robert C. Haynes Jr., E. W. Sutherland, Jr., and T. W. Rall, Western Reserve University School of Medicine, Cleveland

Monday morning, August 31

Cell permeability and hormone action.
O. Hechter and G. Lester, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts

Monday evening, August 31

II. Peptide Hormones

The immunological assay of human pituitary growth hormone.
Charles H. Read and George T. Bryan, State University of Iowa, University Hospital, Iowa City

Tuesday morning, September 1

Studies on erythropoetin.
W. F. White, Clifford W. Gurney and Eugene Goldwasser, Armour and Co. and Argonne Cancer Research Hospital, Chicago

Tuesday morning, September 1

Hormonal activity of active polypeptides.
Hector Croxatto and Livio Barnafi, Catholic University, Santiago, Chile

Tuesday evening, September 1

III. Thyroid Hormones

Studies on the chemistry and bioassay of thyrotropin from bovine pituitaries and transplantable pituitary tumors of mice.

Robert W. Bates and Peter G. Condliffe, National Institutes of Health, Bethesda

Wednesday morning, September 2

The transport of thyroid hormone and its relation to its metabolic fate.
Sidney Ingbar, Thorndike Memorial Laboratory, The Boston City Hospital, Boston

Wednesday morning, September 2

Re-evaluation of some concepts of iodine metabolism.
L. Van Middlesworth, The University of Tennessee, Memphis, Tennessee

Thursday morning, September 3

IV. Hormones and Metabolism

Food and temperature.
John R. Brobeck, University of Pennsylvania School of Medicine, Philadelphia

Thursday morning, September 3

Influence of hormones on adipose tissue as a center of fat transport.
H. E. Wertheimer, The Hebrew University—Hadassah Medical School, Jerusalem, Israel

Thursday evening, September 3

Hormonal control of protein and fat metabolism in the pancreatectomized rat.
Robert O. Scow and Sidney S. Chernick, National Institutes of Health, Bethesda

Friday morning, September 4

Hormonal regulation of hepatic carbohydrate metabolism.
J. Ashmore, Indiana University Medical Center, Indianapolis, Indiana; George Cahill, Jr., New England Deaconess Hospital, Boston; and A. Baird Hastings, Scrips Metabolic Clinic La Jolla, California

Friday morning, September 4

THE AMERICAN GOITER ASSOCIATION

The 1959 meeting of the American Goiter Association will be held in the Drake Hotel, Chicago, Illinois, April 30, May 1 and 2. The program for the three day meeting will consist of papers and discussion dealing with the thyroid gland, its physiology, pharmacology, pathology and therapy.

Secretary
John C. McClintock, M.D.
149½ Washington Ave.
Albany 10, New York

STEROID BIOCHEMISTRY

The fourth session of the Training Program for Steroid Biochemistry sponsored by the U. S. Public Health Service will begin October 1. Applications are invited at the postdoctoral level for a one-year tenure with a stipend of $5500. The Program consists of lectures, planned laboratory, and eight months of research at the Worcester Foundation for Experimental Biology and Clark University or the University of Utah. Inquiries should be addressed to William R. Nes, Department of Chemistry, Clark University, Worcester, Massachusetts, or to Kristen Eik-Nes, College of Medicine, University of Utah, Salt Lake City. A predoctoral program also will be carried out in Worcester with stipends of $1800 for six months. All applications must be received prior to June 1.

THE MYASTHENIA GRAVIS FOUNDATION, INC.

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THE NATIONAL INSTITUTE OF NEUROLOGICAL DISEASES AND BLINDNESS
Bethesda 14, Maryland

April 18 and 19, 1959

LOS ANGELES, CALIFORNIA
THE STATLER-HILTON HOTEL

This Conference will afford the 57 speakers from all of Western Europe, Canada, Soviet Russia and the United States an opportunity to present their current work, discuss mutual problems and enlist suggestions, thereby preventing duplication of efforts.

NEW BOOK REGISTER

PRINCIPLES OF RESEARCH IN BIOLOGY AND MEDICINE. Dwight J. Ingle. Philadelphia: J. B. Lippincott Co. 123 pages. $4.75

ALDOSTERONE. An international symposium edited by Alex F. Muller and Cecilia M. O'Connor. In this symposium the biogenesis and its importance in clinical medicine are critically evaluated in light of our present knowledge. Boston: Little, Brown & Co. 232 pages. $8.00

THE LIPOPROTEINS METHODS AND CLINICAL SIGNIFICANCE. Edited by F. Homburger and P. Bernfield, Basel. New York: S. Karger. 94 pages. $4.00

DIE MEDIKAMENTÖSE BEHANDLUNG DER SCHILDDRÜSENERKRANKUNGEN. W. Grab and K. Oberdisse. Stuttgart: Georg Thieme Verlag. 285 pages; 55 illustrations. $9.00

KLINISCHE ENZYMOLOGIE. Rudolf Abderhalden. Stuttgart: George Thieme Verlag. 390 pages; 34 illustrations. $11.40

HORMONII STEROIZI. A. Lupulescu. Bucuresti: Editura Medicala. 400 pages; 146 illustrations.

A DOCTOR SPEAKS HIS MIND. Roger I. Lee. Boston: Little, Brown & Co. 120 pages. $3.50

CLINICAL ENZYMOLOGY. Edited by Gustav J. Martin. Seven contributors. This book discusses a new field of medicine—the parenteral administration of enzymes, their systemic actions and their therapeutic effects. Boston: Little, Brown and Co. 241 pages. $6.00

THE ENDOCRINOLOGY OF REPRODUCTION. Joseph Thomas Velardo, Editor. Eight authors. Within recent years numerous researches have been conducted in an effort to elucidate the action of the hormones on the physiology of the reproductive organs. This new work has been prepared to serve an area where previously there was no adequate textbook. Its purpose is not only to furnish the student a statement of our present knowledge concerning the endocrinology of reproduction but also to indicate the direction that our further research must take. New York: Oxford University Press. Illustrated. 340 pages. $15.00

ESSENTIALS OF HUMAN REPRODUCTION. Joseph Thomas Velardo, Editor. Thirteen authors. It has been almost twenty years since a definitive account of the essentials of human reproduction has been related in textbook form. This volume, written by a group of leading specialists in clinical endocrinology, has been prepared to fill this vital need. It is the first book ever written that explores the whole life history of the reproductive process from ovulation through pregnancy. New York: Oxford University Press. Illustrated. 270 pages. $13.50

METABOLIC DISTURBANCES IN CLINICAL MEDICINE. Edited by G. A. Smart. Fourteen contributors. This book is designed to give definite help in diagnosis and treatment of metabolic diseases. Boston: Little, Brown & Co. Illustrated. 358 pages. $10.00

ETIOLOGY AND TREATMENT OF LEUKEMIA. Proceedings of the First Louisiana Cancer Conference edited by Walter J. Burdette. St. Louis: The C. V. Mosby Co. 167 pages. $4.00

THE CEREBROSPINAL FLUID. A Ciba Foundation Symposium. Anatomists, neurologists, pathologists, psychiatrists, pharmacologists and neurosurgeons came from all over the world to attend this meeting to contribute the distilled products of their research and reflections. Boston: Little, Brown & Co. 335 pages. $9.00

HORMONE PRODUCTION IN ENDOCRINE TUMOURS. A Ciba Foundation colloquia on Endocrinology. Another important addition to the endocrinology series, is concerned with hormone-producing tumors, the functional tumors of the endocrine system, and a summary of the important work done in this field during the last twenty-five years. Boston: Little, Brown & Co. 351 pages. $9.00

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WATER AND ELECTROLYTE METABOLISM IN RELATION TO AGE AND SEX: A Ciba Foundation Colloquia on Ageing. This volume contains the proceedings of the fourth colloquium in this series sponsored by the Ciba Foundation. With the realization that electrolytes enter into practically every reaction that takes place in the body and that we still know very little about many of these, the Ciba Foundation asked experts on all aspects of this subject to share their ideas and experiences in an attempt to throw more light on these unknowns. The program was divided to cover general principles, the developing organism and senescence and disease. Boston: Little, Brown & Co. 327 pages. $8.50

THE BIOLOGY OF HAIR GROWTH. This book, written by twenty-one scientists, presents an up-to-date account of the organization, differentiation, and growth of hair. New York: Academic Press Inc. 520 pages. $15.00

L'ADRENALINE ET LA NORADRENALINE DANS LA REGULATION DES FONCTIONS HOMEOSTASIQUES. Colloques Nationaux de Centre National de la Recherche Scientifique. 250 pages.

THE CHEMICAL PREVENTION OF CARDIAC NECROSES. This book provides a complete synthesis of the current clinical and experimental observations on cardiac necroses. This information is viewed in the light of newly acquired knowledge about the production and prevention of the electrolyte-steroid-stress cardiopathies in animals. New York: The Ronald Press Co. 235 pages. $7.50

THE HEALING OF WOUNDS. A symposium on recent trends and studies. The work integrates in one single volume the wound healing knowledge and experience of men noted in such diverse fields as surgery, biochemistry, anatomy, obstetrics, gynecology, and pharmacology. 202 pages. $7.00

CLINICAL OBSTETRICS AND GYNECOLOGY. Volume 1, No. 4. Symposium on Operative Obstetrics, J. Robert Willson, M. D., guest editor and Symposium on Genital Cancer, Daniel G. Morton, M. D., guest editor. New York: Paul B. Hoeber. $18.00 per year.

REPRODUCTIVE PHYSIOLOGY. Comparative reproductive physiology of domestic animals, laboratory animals and man. A. V. Nalbandov. San Francisco: W. H. Freeman & Co. 271 pages; illustrated. $6.75

CHLOROMYCETIN. Theodore E. Woodward and Charles L. Wisseman. Antibiotics monograph No. 8. New York: Medical Encyclopedia, Inc. 152 pages. $4.00

PENICILLIN. Harold L. Hirsh and Lawrence E. Putman. Antibiotics monogrpah No. 9. New York: Medical Encyclopedia, Inc. 148 pages. $4.00

STREPTOMYCIN AND DIHYDROSTREPTOMYCIN. Louis Weinstein and N. Joel Ehrenkranz. Antibiotics monographs No. 10. New York Medical Encyclopedia, Inc. 116 pages. $4.00

MODERN CHEMOTHERAPY OF TUBERCULOSIS. Roger S. Mitchell and J. Carroll Bell. Antibiotics monographs No. 11. New York: Medical Encyclopedia, Inc. 109 pages. $4.00